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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/015,948	12/11/2001	Keith D. Allen	R-605	2942

7590 11/19/2003

DELTAGEN, INC.
740 Bay Road
Redwood City, CA 94063

EXAMINER	
TON, THAIAN N	

ART UNIT	PAPER NUMBER
1632	

DATE MAILED: 11/19/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/015,948	ALLEN ET AL.	
	Examiner	Art Unit	
	Thái-An N. Ton	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *31 July 2003* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-38* is/are pending in the application.

4a) Of the above claim(s) *1, 2, 10-13, 29-38* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *3-9 and 14-28* is/are rejected.

7)☒ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *11 December 2001* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s) *7/9/02* .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

Claims 1-38 are pending. Claims 1, 2, 10-13 and 29-38 are withdrawn. Claims 3-9 and 14-28 are under current examination.

Election/Restrictions

Claims 1, 2, 10-13 and 29-38 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected groups, there being no allowable generic or linking claim. Election was made **without** traverse in the paper filed 7/31/03.

Applicant's election without traverse of Group II [claims 3-9 and 14-28] in the paper filed 7/31/03 is acknowledged.

Information Disclosure Statement

The Information Disclosure Statement, filed July 9, 2002, has been considered.

Claim Objections

Claim 9 is objected to because the claim is dependent upon a non-elected claim. The claim should be written in an independent form.

Claim Rejections - 35 USC § 101/112

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 3-9 and 14-28 are rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a specific, substantial and credible asserted utility or a well-established utility.

The claims are directed to transgenic mice and methods of producing transgenic mice comprising a disruption in an adrenocorticotropin receptor [ACTHR] gene and cells derived from the transgenic mice.

The specification teaches methods of generation knockout mice and cells comprising a disruption in the ACTHR gene. The knockout mice generated by introducing a ACTHR targeting construct into mouse ES cells to generate chimeric mice, which were then bred to produce heterozygotes which were then backcrossed to generate ACTHR homozygous knockout mice. See Example 1. The specification teaches that the homozygous knockout mice of the invention exhibit a phenotype of hypoplasia of the adrenal gland, abnormalities in brown adipose tissue [particularly decreased cytoplasmic lipid vacuolation of brown adipose tissue], decreased body fat percentage, an increased susceptibility to seizure, hyperactivity, and an anti-

depressant phenotype, as seen by a decrease in total time spent immobile in the tail suspension test. See pp. 3-4 and Examples 2-5 of the specification. The specification teaches that the cell- and animal-based systems can be used as models for diseases and then used in assays to screen strategies designed to identify agents, such as compounds capable of ameliorating disease symptoms. See p. 19, lines 19-27.

The specification has provided general teachings that the claimed transgenic mice may be used to identify agents that affect a phenotype related to the mice [see p. 20, lines 16-20, for example. As such, the asserted utility of the claimed transgenic mice is for screening agents that may affect the phenotype of hypoplasia of the adrenal gland, abnormalities in brown adipose tissue [particularly decreased cytoplasmic lipid vacuolation of brown adipose tissue], decreased body fat percentage, an increased susceptibility to seizure, hyperactivity, and an anti-depressant phenotype, as seen by a decrease in total time spent immobile in the tail suspension test. The asserted utility does not appear credible to the skilled artisan because the evidence of record has not provided a correlation between an ACTHR gene and these phenotypes and any disease or disorder. As the evidence of record has not provided a correlation between these phenotypes and any disease or disorder, the utility of identifying agents that affect the described phenotypes is not apparent. Furthermore, the evidence of record has not provided any other utilities for the claimed transgenic mouse that are specific, substantial and credible.

The specification teaches that the asserted utility of the claimed transgenic mouse and cells derived therefrom would be as a model for disease. The specification fails to correlate the phenotype [hypoplasia of the adrenal gland, abnormalities in brown adipose tissue [particularly decreased cytoplasmic lipid vacuolation of brown adipose tissue], decreased body fat percentage, an increased susceptibility to seizure, hyperactivity, and an anti-depressant phenotype, as seen by a decrease in total time spent immobile in the tail suspension test] of the claimed transgenic mice with any disease or disorder. These phenotypes are not found to be associated with any disease or condition.

Furthermore, the specification and art teach that mutations in ACTHR result in FGD. Weber *et al.* [Reference 7 of Applicants' IDS filed 7/9/02] teach that FGD is, "[C]haracterized by the presence of elevated circulating ACTH levels, but normal mineralocorticoid production. Patients usually present in early childhood with one or more of the following: hyperpigmentation, hypoglycemic episodes, failure to thrive, and frequent and severe infections. However, age of onset of symptoms and clinical severity of the disease vary considerably between cases, suggesting a heterogeneous genetic origin." See p. 65, 1st column, 1st ¶. If Applicants intend for the claimed transgenic mice to be disease models for FGD, there is no correlation or nexus provided by the specification to show that the mice exhibit any phenotype associated with FGD.

Accordingly, neither the specification, nor any evidence of record, provide a correlation or nexus between the phenotypes associated with the homozygous knockout ACTH mice and any disease or disorder, leaving the skilled artisan to speculate and investigate further uses for the claimed transgenic mice.

Claims 3-9 and 14-28 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Specifically, since the claimed invention is not supported by either a specific, substantial and credible asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention, claims 3-9 and 14-28 lack enablement.

Furthermore, the mere capability to perform gene transfer in a mouse is not enabling because a desired phenotype cannot be predictably achieved by simply introducing transgene constructs of the types recited in the claims. While gene transfer techniques are well developed for a number of species, and in particular, the mouse, methods for achieving the desired level of transgene expression in appropriate tissues are less well established. The introduction of DNA into the mammalian genome can ordinarily be achieved most reliably by microinjection or

retrovirus-mediated gene transfer. However, the state of the art for transgenics is unpredictable because the method of gene transfer typically relies on random integration of the transgene construct. Insertional inactivation of endogenous genes and position effects [see Ryan *et al.*, **Sem. Neph.** 22:154-160, 2002] can dramatically influence the phenotype of the resultant transgenic animal. Ryan *et al.* state that methods such as pronuclear injection or gene targeting by homologous recombination are still limited by several unpredictabilities, including differences in transgene copy number and position of integration into the genome. Furthermore, Ryan *et al.* state "The location of integration can have dramatic effects on the expression of a transgene. Called the position effect, transcriptional regulatory sequences at or near the insertion site can strongly influence your transgene, even impart a new set of instructions. " [See p. 155, 2nd column].

Additionally, the state of the art of generation of knockout animals is found to be unpredictable. For example, the knockout art teaches that the disruption of a different exon of the same gene may **not** result in the anticipated phenotype. See Moreadith et al. (Journal of Molecular Medicine, 1997) who support phenotypic unpredictability in knockout mice. In particular, Moreadith et al. discuss that gene targeting at a particular loci is unpredictable with respect to the resulting phenotype since often the generation of knockout mice, in many instances, changes the prevailing notions regarding the functions of the encoded proteins. For example, Moreadith et al. report that gene targeting at the endothelin loci led to the

creation of mice with Hirschsprung's disease instead of the anticipated phenotype (abnormal control of blood pressure). See page 208, column 2, 2nd paragraph.

The breadth of the claimed invention encompasses chimeric, heterozygous and homozygous animals. The specification teaches homozygous ACTHR knockout mice that exhibit a phenotype of hypoplasia of the adrenal gland, abnormalities in brown adipose tissue [particularly decreased cytoplasmic lipid vacuolation of brown adipose tissue], decreased body fat percentage, an increased susceptibility to seizure, hyperactivity, and an anti-depressant phenotype, as seen by a decrease in total time spent immobile in the tail suspension test. The specification teaches that the animals of the instant invention can be used as models for diseases. See p. 19, lines 19-27. However, the specification fails to teach a phenotype associated with the chimeric or heterozygous animals comprising a disruption in an ACTHR gene, as encompassed by the claims. As such, one of skill in the art would not know how to use the chimeric mice or heterozygous mice as encompassed by the claims because they have no disclosed phenotype that would make them useful as disease models.

Given that specific phenotypic alterations cannot be predictably achieved by merely transferring a gene of interest into an animal, specific guidance must be provided to enable the instant invention. The specification must teach those skilled in the art how to make and use the full scope of the instant invention. The specification teaches that the claimed transgenic non-human animals, and

transgenic mice would be used as models for disease [see p. 19, lines 19-27]; however, the specification does not enable this use. In absence of a disclosure of a transgenic animal exhibiting an appropriate phenotype, undue experimentation would have been required to make and use the claimed transgenic non-human animals. The specification specifically discloses the disruption of the ACTHR gene and that the transgenic animals of the invention may be used as models for diseases, disorders, or conditions associated with phenotypes relating to a disruption in the ACTHR gene [see pp. 20-21, bridging sentence]; however, the phenotypes as taught by the specification do not correlate to an ACTHR disease.

The breadth of the claimed invention is directed to the generation of transgenic non-human animals comprising a targeted disruption in the ACTHR gene, which requires embryonic stem [ES] cells. The state of the art is such that ES cell technology is generally limited to the mouse system at present, and that only "putative" ES cells exist for other species (see Moreadith *et al.*, **J. Mol. Med.**, 1997, p. 214, S*ummary*). Note that "putative" ES cells lack a demonstration of the cell to give rise to germline tissue or the whole animal, a demonstration which is an art-recognized property of ES cells. Moreadith *et al.* supports this observation as they discuss the historical perspective of mouse ES cells as follows:

"The stage was set-one could grow normal, diploid ES cells in culture for multiple passages without loss of the ability to contribute to normal development. Furthermore, the cells contributed to the development of gametes at a high

frequency (germline competence) and the haploid genomes of these cells were transmitted to the next generation. Thus, the introduction of mutations in these cells offered the possibility of producing mice with a predetermined genotype."

Such a demonstration has not been provided by the specification or the prior art with regard to the generation of any species of animal ES cells, other than the mouse, which can give rise to the germline tissue of a developing animal. In addition, prior to the time of filing, Mullins *et al.* (**Journal of Clinical Investigation**, 1996) report that "although to date chimeric animals have been generated from several species including the pig, in no species other than the mouse has germline transmission of an ES cell been successfully demonstrated." (page 1558, column 2, first paragraph). As the claims are drawn to methods involving the manipulation of animal embryonic stem (ES), and particularly since the subject matter of the specification and the claimed invention encompasses the use of such cells for the generation of a transgenic animal, the state of the art supports that only mouse ES cells were available for use for production of transgenic mice.

Note that claim 5 is directed to a murine ES cell. The term "murine" encompasses both mice and rats. As stated above, the state of the art only supports that mouse ES cells were available for the generation of knockout mice.

This is further supported by Pera *et al.* [**Journal of Cell Science** 113: 5-10 (2000)] who present the generic criteria for pluripotent ES or EG cells [see p. 6, 2nd

column] and state that, "Thus far, only mouse EG or ES cells meet these generic criteria." [See p. 6, 2nd column, last paragraph].

Accordingly, in view of the quantity of experimentation necessary for the production and use of non-human transgenic animals comprising a disruption in an ACTHR gene, the lack of direction or guidance, as well as working examples, provided by the specification for the production and use of non-human transgenic animals, for the breadth claimed, the unpredictable and undeveloped state of the art for the production of transgenic knockout non-human animals, particularly with respect to the unpredictable nature of the phenotypic effect, and the breadth of the claims encompassing all non-human animals, it would have required undue experimentation for one of skill in the art to make and use the claimed non-human transgenic animals, cells, and methods of using the same.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the

subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 3-9, 14 and 28 are rejected under 35 U.S.C. 103(a) as being unpatentable over Capecchi [**Scientific American**, 1994, 270:34-41] when taken with Kubo *et al.* [Reference 1 of Applicant's IDS filed 7/9/02].

Capecchi teaches knockout technology applied to mice, specifically with respect to the disruption of the *Hox*A-3 gene and as a method of producing the same, applies to determining the *in vivo* biological function of any known gene of interest [see p. 37, col. 1-2, bridging ¶ and Figures pp. 36-37]. For example, Capecchi discloses the applicability of gene targeting to many other genes, so that a correlation can be drawn between the malfunctioning gene to the manifestation of disease [see p. 41, col. 2, 2nd full paragraph]. Capecchi further discloses the essential components of a targeting vector [p. 38, col. 3, and p. 39, col. 1-2], and the steps involved for targeted gene replacement in ES cells as well as in mice [see p. 36-39 and diagrams]. Capecchi differs from the claimed invention in that the targeting construct does not contain flanking nucleotide sequences which

homologous recombine with the mouse ACTHR gene. However, prior to the time the claimed invention was made, Kubo teach the cloning of the mouse ACTHR gene. See Figure 1.

Note that absent any phenotypic requirements of the claimed transgenic mice, the combination of the cited prior art is sufficient to make obvious the invention. Further note that it would be well-known in the art that the disruption of any gene of interest, at any particular exon would have a reasonable expectation of success in the decreased expression of that particular gene.

Accordingly, in view of the combined teachings, it would have been obvious for one of ordinary skill in the art, at the time the claimed invention was made, to modify the knockout technology of Capecchi by use of a targeting vector for the disruption of the known mouse ACTHR gene in a mouse with a reasonable expectation of success. One of ordinary skill would have been sufficiently motivated to make such a modification, as it was an art-recognized goal to determine the physiological role of a gene of interest by the generation of a knockout mouse, as supported by Capecchi who teach that the generation of mouse models will allow for the observation of effect of a knocking out a particular gene on disease phenotypes. See p. 41, 2nd column, 2nd ¶.

Thus the claimed invention as a whole was clearly *prima facie* obvious at the time the claimed invention was made especially in the absence of sufficient, clear and convincing evidence to the contrary.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Thái-An N. Ton whose telephone number is (703) 305-1019. The examiner can normally be reached on Monday through Friday from 8:00 to 5:00 (Eastern Standard Time), with alternating Fridays off. Should the examiner be unavailable, inquiries should be directed to Deborah Reynolds, Supervisory Primary Examiner of Art Unit 1632, at (703) 305-4051. Any administrative or procedural questions should be directed to William Phillips, Patent Analyst, at (703) 305-3482. Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Fax Center number is (703)-872-9306.

TNT
Thái-An N. Ton
Patent Examiner
Group 1632

Deborah Crouch

DEBORAH CROUCH
PRIMARY EXAMINER
GROUP ~~1800~~ 1630

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	10/015,948
Filing Date	12/11/01
First Named Inventor	Keith D. Allen, et al.
Art Unit	~~1642~~ 1652
Examiner Name	~~Unassigned~~ TON
Attorney Docket Number	R-605

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OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T [2]
TNT	1	MITSUMASA KUBO, et al. "Cloning of a mouse adrenocorticotropin receptor-encoding gene *"*Gene*, Vol. 153 (1995), pp. 279-280	
	2	MARIE-CHRISTINE LEBRETHON, et al. "Regulation of Corticotropin Recepotor Number and Messenger RNA in Cultured Human Andrenocortical Cells by Corticotropin and Angiotensin II" *J. Clin. Invest.*, Vol. 93, 04/94, pp. 1828-1833	
	3	KATHLEEN G. MOUNTJOY, et al. "The Cloning of a Family of Genes That Encode the Melanocortin Receptors" *Science*, Vol. 257, 08/28/92, pp. 1248-1251	
	4	DANIELLE NAVILLE, et al. "Genomic Structure and Promoter Characterization of the Human ACTH Receptor Gene" *Biochemical and Biophysical Research Communications*, Vol. 230, (1997), pp. 7-12	
	5	J. RAMACHANDRAN, "Corticotropin Receptors, Cyclic AMP and Steroidogenesis" *Endocrine Research, Vol. 10(3&4)*, (1985), pp. 247-363	
	6	BERNARD P. SCHIMMER, "Cyclic Nucleotides in Hormonal Regulation of Adrenocortical Function" *Advances in Cyclic Nucleotide Research*, Vol. 13, (1980), pp. 181-214	
TNT	7	ANGELA WEBER, et al., "Adrenocorticotropin Receptor Gene Mutations in Familial Glucocorticoid Deficiency: Relationships with Clinical Features in Four Families"*Journal of Clinical Endocrinology and Metabolism*, Vol. 80(1) (1995), pp. 65-71	

Examiner Signature		Date Considered	11/5/03

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

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	Examiner Thái-An N. Ton	Art Unit 1632	Page 1 of 2

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	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Moreadith et al. Journal of Molecular Medicine, 1997.
	V	Mullins, L.J. et al. J. Clin. Invest., 98(11):S37-S40 (1996).
	W	Pera et al. Journal of Cell Science 113: 5-10 (2000).
	X	Capecchi. Scientific American, 1994, 270:34-41.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Notice of References Cited	Application/Control No. 10/015,948	Applicant(s)/Patent Under Reexamination ALLEN ET AL.	
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NON-PATENT DOCUMENTS

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	U	Ryan et al., Sem. Neph. 22:154-160, 2002.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

J Mol Med (1997) 75:208–216

REVIEW

Randall Wade Moreadith · Nina Butwell Radford

Gene targeting in embryonic stem cells: the new physiology and metabolism

Received: 28 June 1996 / Accepted: 4 October 1996

Abstract The development of transgenic technology, whereby genes (or mutations) can be stably introduced into the germline of experimental mammals, now allows investigators to create mice of virtually any genotype and to assess the consequences of these mutations in the context of a developing and intact mammal. In contrast to traditional "gain-of-function" mutations, typically created by microinjection of the gene of interest into the one-celled zygote, gene targeting via homologous recombination in pluripotential embryonic stem cells allows one to modify *precisely* the gene of interest. The purpose of this review is to introduce the reader to the history of development of embryonic stem cell technology, the current methods employed to create "knock-out" mice, and the application of these methods to solve problems in biology. While the technology promises to provide enormous insight into mammalian development genetics, our desire is that this review will stimulate the application of gene targeting in embryonic stem cells to begin to unravel problems in complex regulatory pathways, specifically intermediary metabolism and physiology.

Key words Gene targeting · Transgenic animals · Molecular biology · Metabolism · Physiology

Abbreviations *EC cell* Embryonal carcinoma cell · *ES cell* Embryonic stem cell · *HPRT* Hypoxanthine phosphoribosyltransferase · *NMR* Nuclear magnetic resonance

Introduction

The development of techniques for introducing genes (or mutations) stably into the germline of experimental mammals [1], referred to as "transgenic technology," has provided unique insight into complex biologic phenomena. Although simplistic, this technology can now be broadly defined into two experimental categories: "gain-of-function" mutations, typically created by microinjection of the gene (transgene) of interest directly into the zygote stage of development (for example, to define the controlling elements for a muscle-specific gene), and "loss-of-function" mutations, which employ embryonic stem (ES) cells. The technique of DNA microinjection results in *random integration* of the transgene, giving rise to the founder animal(s). The founder animals are then bred individually to establish independent lines of transgenic animals that can undergo further characterization [e.g., pattern(s), levels and consequences of expression of the transgene]. However, many of these lines exhibit variable expression of the gene of interest since the transgene may integrate in a manner that alters its expression (for example, by integration near controlling elements that affect the pattern and level of expression), and this may confound the interpretation of results.

In contrast, the development of gene targeting via homologous recombination in pluripotential ES cells allows one to modify *precisely* the gene of interest. It is now possible to create mice of virtually any genotype, and to assess the consequences of these mutations in the context of a developing and intact mammal. This technology, which is typically used to create the null genotype ("knock-out" mice), has frequently provided the definitive experimental evidence regarding the functions of the encoded proteins. However, in many instances these mutations have changed the prevailing notions. For example, gene targeting at the endothelin loci subsequently led to the creation of mice with Hirschsprung's disease (aganglionic megacolon [2]) instead of the anticipated phenotype (abnormal control of blood pressure). Indeed, if one had even predicted these mice would survive the absence of a cellular gene that is so widely expressed, one might have been in the minority!

The purpose of this review is to introduce the reader to the history of development of ES technology, the cur-

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rent methods employed to create "knock-out" mice, and the application of these methods to solve problems in biology. While the technology promises to provide enormous insight into mammalian development genetics, our desire is that this review will stimulate the application of gene targeting in ES cells to begin to unravel problems in complex regulatory pathways, specifically intermediary metabolism and physiology.

Historical development

Derivation and characterization of pluripotential stem cells

The development of gene-targeting technology in ES cells is an example of the convergence of classical cell biology with molecular biology. One of the seminal papers was published in 1975 by Mintz and colleagues at the Fox Chase Cancer Center [3]. It was well established that explantation of normal mouse embryos into extrauterine sites (typically the kidney capsule) subsequently led to the development of tumors known as teratocarcinomas. Cell lines could be derived from the tumors, passaged indefinitely in tissue culture, and when reintroduced into recipient mice gave rise to tumors with cell types representative of all germ layers (ectoderm, mesoderm, and endoderm). These properties suggested that these embryonal carcinoma (EC) cells were pluripotential, that is, capable of differentiating into a host of cell types under the appropriate conditions. In many instances these tumors contained cell types indistinguishable from the normal cellular counterpart in biochemical or ultrastructural detail. Thus, although these cells were considered to be "highly" malignant, it was quite clear that not all tissues derived from them displayed the malignant properties of the parental cells. Mintz and colleagues reasoned that these EC cells might in fact be totipotential – capable of contributing to the development of all *normal* tissues under the appropriate environmental cues "For this to occur, the initially malignant cells would presumably have to be brought into association with early embryo cells so that the latter could provide an organizational framework appropriate for normal development [3]." This was an astounding leap of logic at the time. She and her colleagues subsequently demonstrated that malignant EC cells, grown as an ascites tumor for over 8 years, could give rise to apparently normal mosaic mice upon introduction into normal blastocysts. Furthermore, the mosaic mice were capable of transmitting the EC cell genotype through the germline, that is, mating of these mosaic mice with wild-type partners gave rise to mice that were genotypically derived from the EC cells! These results, and those from many other laboratories, established the experimental basis for subsequent developments in the field. Indeed, Mintz precisely predicted the emergence of stem cell technology. "Thus, EC cells ... offer new possibilities for studying mammalian regulatory systems: the carcinoma cells could first be experimentally mutagenized and selected during a brief in vitro sojourn and then cycled through mice via blastocyst injections. Participation in differentiation of a mosaic individual would permit developmental and biochemical analyses of the mutations; conversion of some cells to gametes would enable genetic analysis and mapping of the mutated regions through recombination and segregation during meiosis [3]."

These observations, made prior to the large-scale development of classical molecular biology (note that the above experiments were published prior to the development of DNA sequencing!) were quickly verified by many laboratories. However, working with EC cells proved to be quite cumbersome due to their propensity for aneuploidy, and the attention turned to isolation of pluripotential cells from *normal* embryos. Early attempts failed until 1981, when Evans and Kaufman [4] and Martin [5] independently described the growth and maintenance of euploid cells, derived from normal mouse embryos explanted in culture, that displayed pluripotential properties (hereafter referred to as ES cells). This was quickly followed by the demonstration in 1984 these cells were capable of giving rise to germline chimeras upon introduction into normal blastocysts [6]. The stage was set – one could grow normal, diploid ES cells in culture for multiple passages without loss of the ability to contribute to normal development. Furthermore, the cells contributed to the development of gametes at a high frequency (germline competence), and the haploid genomes of these cells were transmitted to the next generation. Thus, introduction of mutations in these cells offered the possibility of producing mice with a predetermined genotype.

Homologous recombination: introduction of precise mutations into resident genes

In an elegant series of papers using mammalian cell lines [7–10], based primarily on prior work in *Saccharomyces cerrivisiae*, it became clear that cloned DNA could be precisely altered in vitro, and when introduced into cells via a number of methods (infection, transfection) would homologously recombine with the resident gene and introduce the desired mutation at that site in the genome. In lower eukaryotes (yeast and *Neurospora*) recombination at the homologous locus is the favored reaction and can occur essentially without selection, but in higher eukaryotes (mouse and *Drosophila*) the frequency of homologous recombination is rare. This necessitates the use of *positive* selection; typically, these rare events are selected for by introduction of genes conveying resistance to otherwise toxic metabolites (hygromycin, neomycin). Note that the introduction of a positive selection marker gene is also frequently used to disrupt (mutate) the gene of interest. In addition, one can enhance the frequency of these events by employing the additional strategy of *negative* selection – most commonly performed by use of the thymidine kinase (*TK*) gene flanking the targeting vector. ES

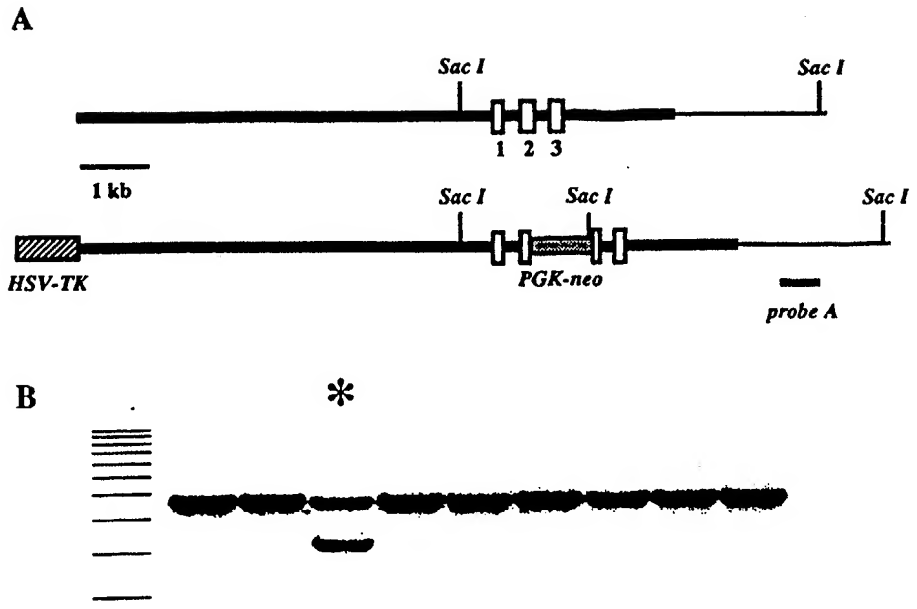

Fig. 1A, B Gene targeting at the murine cytochrome oxidase VIaH locus. **A** The structure of the gene, which has been reported elsewhere [12]. Briefly, the gene is comprised of three small exons (*open rectangles*). Exon 2 was disrupted with the expression casette for neomycin phosphotransferase (*PGK-neo*), thus mutating the gene in its coding region and simultaneously providing positive selection. *Heavy lines*, the cloned vector used for transfection into J1 ES cells; note the targeting vector is flanked with the expression casette for TK (*HSV-TK*) to allow for negative selection (see text). **B** Probe A was derived from a separate region of the gene (*thin line*) and was used to screen, by Southern analysis, DNA isolated from G418- and gancyclovir-resistant ES clones. Note the presence of the endogenous band at 5.7 kb in all clones, as well as the presence of the recombinant band at 4.2 kb (*asterisk*), in a *Sac*I digest of genomic DNA. Clone 3 was then used for blastocyst injection as illustrated in Fig. 2 to generate germline chimeras. *Bars* (*left*), 1-kb-size markers

cell clones which retain the *TK* gene (nonhomologous recombination) do not survive the addition of gancyclovir (or homologues) to the media due to the accumulation of toxic nucleosides, whereas cell clones which have undergone an authentic recombination lose the *TK* gene. This combined strategy is known as *positive-negative* selection [11] and routinely increases the targeting frequency by an order of magnitude or more.

An example of gene targeting in ES cells is illustrated in Fig. 1. The gene of interest in this example is a muscle-specific subunit of cytochrome oxidase (VIaH). The structure and regulation of the gene have recently been defined in the author's laboratory [12], but the precise function of this subunit, postulated to regulate the steady-state activity of cytochrome oxidase, remains largely unknown in the context of an intact animal. It is anticipated that creation of mice which lack this subunit will provide more insight into the precise role this subunit may play in the bioenergetics of cardiac and skeletal muscle.

Once these mutated ES cells are isolated as a pure clone, they can be introduced into the blastocoele cavity of a normal embryo, where they participate in the development of all tissues and result in the production of chimeras (usually assessed at birth by acquisition of the dominant coat color phenotype Agouti). In subsequent matings of these chimeric mice, if the ES cells have contributed to formation of germ cells, the mutant gene is transmitted to their progeny. By mating heterozygotes, each harboring a mutated copy of the gene of interest (detected by analysis of the isolated DNA), one can derive embryos and mice which are homozygous for the mutation. These techniques are illustrated schematically in Fig. 2.

"Knockout mice" : the new genetics

The initial attempts at gene inactivation in murine ES cells took advantage of selection methods designed to reflect loss of an enzyme activity, hypoxanthine phosphoribosyltransferase (HPRT), following random integration of proviruses [13, 14]. Since the vast majority of ES cell lines used were male (male chimeric mice can be mated frequently, thus producing numerous offspring to assess germline competence), random integration of DNA (in this case retroviruses) would be expected to inactivate the single copy of the HPRT gene on the X chromosome at a low but detectable frequency and subsequently confer growth properties in a defined (hypoxanthine-aminopterin-thymidine) medium. These HPRT-minus cell lines were used to make mice deficient in HPRT, a potential mouse model of the human disorder Lesch-Nyhan syndrome [14, 15]. However, the absence of a neurologic phenotype in these mice was readily apparent, eventually precipitating a search for alternative explanations of why purine salvage might be different in the two species. This lead to the elucidation that adenine phosphoribosyltransferase was the major enzyme involved in purine salvage in the mouse. Indeed, when HPRT-deficient mice were exposed to inhibitors of adenine phosphoribosyltransfer-

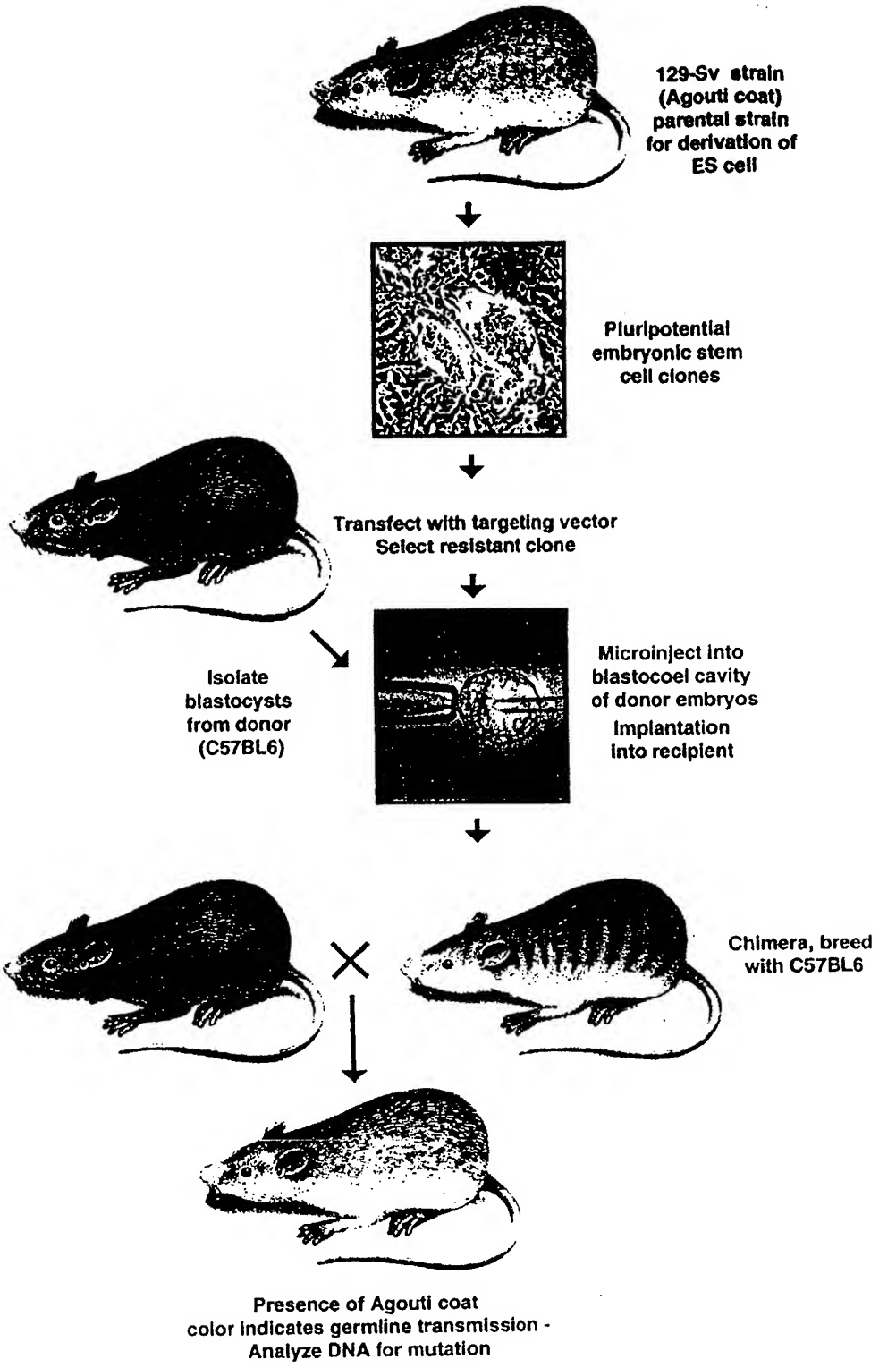

Fig. 2 General strategy for creating "knock-out" mice

ase, they developed neurologic phenotypes more representative of the human disorder [16]. The HPRT locus continued to be the subject of intense investigation, however, defining many of the parameters routinely employed now to target genes – for example, length and degree of homology to mediate highly efficient gene targeting [17, 18].

The first description of gene targeting via homologous recombination in murine ES cells was published in 1987 [19]. To date, several hundred novel mouse mutants – "knockout" mice – have been created, with dozens being reported monthly. A synopsis of these mutations is obviously beyond the scope of this review, but it is readily apparent from a perusal of these mutations that the

Table 1 Established methodologies for the study of murine physiology and metabolism

Methodology	Physiologic measure	References
Cardiovascular system		
Micromanometer catheter	LV hemodynamics	22–24
Indwelling arterial catheters	Blood pressure, heart rate	25–28
Reference microsphere and dilution	Cardiac output, regional blood flows, intravascular fluid volumes	26, 28
Echocardiography	LV mass, LV systolic function, wall motion abnormalities, heart rate	29–32
X-ray contrast microangiography	Ventricular volumes, ejection fraction	33
Swimming	Cardiac adaptations to chronic exercise	34
Langendorff perfusion	Intraventricular pressure, indices of LV contraction and relaxation, heart rate	35–37
Magnetic resonance imaging	Cardiac chamber sizes, coronary flow	38, 39
Magnetic resonance spectroscopy	pH, high energy phosphates	40
Tail-cuff sphygmomanometer	Blood pressure	27
Pulmonary system		
Plethysmography	Lung volumes, respiratory rate	41–44
Pulse oximetry	Arterial saturation	45
Pressure-volume curves	Elastic recoil	46
Forced oscillation	Pulmonary resistance	46
Methacholine challenge	Airway responsiveness	47
Skeletal muscle		
Skeletal muscle resection	Response to mechanical overload	48, 49
Magnetic resonance spectroscopy	pH, phosphocreatine, ATP, inorganic Phosphate, creatine kinase flux	50, 51
Microvascular flow	Ischemia/reperfusion injury	49, 52, 53
Programmable treadmill	Fatiguability	54
Miscellaneous		55
Magnetic resonance imaging	Knee joint degeneration	56
	Polycystic kidneys	57
Magentic resonance spectroscopy	Renal edema, hepatic iron deposition	58
	Hepatic ADP levels, creatine kinase flux	59
	Brain pH, free Mg, choline, N-acetylaspartate, creatine	60
Pathogen inoculation	Phagocyte oxidase function	61
Morris Water maze task, visible or hidden-platform tasks	Spatial learning	62, 63
Intruder test	Defensive aggression	64
Contextural and tone conditioning	Fear response	64
Swimming	Exercise-induced immunosuppression	65

technology has become one of the most powerful methods in the repertoire of approaches to gain insight into the functions of genes. To cite only a few applications, these include developmental biology, behavior and cognition, pharmaceutical research, and generation of models of human disease, such as cystic fibrosis and familial hypercholesterolemia [20, 21].

The new physiology and metabolism

Gene targeting in ES cells has only recently been applied to address problems in classical physiology and metabolism. Indeed, the creation of mutant animals, some of which have unpredictable and subtle phenotypes, has rekindled interest in developing techniques that allow one to characterize the animals precisely. This initiative, "molecular physiology," represents a new field of biology that addresses physiology and metabolism in the context of an intact animal harboring defined mutations in selected genes.

Established methodologies, such as light, immunofluorescence, and electron microscopy, are frequently used in postmortem tissue to describe histologic and ultrastructural changes in organs of interest in particular transgenic models of disease. Functional studies in the isolated, intact organ have also been accomplished, but it is becoming increasingly clear that studies of physiology in the intact animal may yield the greatest insight. A summary of methodologies which have been adapted to study murine cardiovascular, respiratory, and skeletal muscle physiology in vitro and in vivo is presented in Table 1. The remainder of our discussion focuses on examples of methodologies which have been developed to interrogate primarily cardiovascular phenotypes. Although most of these examples represent gain-of-function mutations, it is clear the methods can be applied to loss-of-function mutations as well.

Cardiovascular "molecular physiology"

At the present time ES cell technology exists only in the mouse (although see discussion below); thus creation of animals with anticipated cardiac phenotypes requires that

techniques be developed to interrogate them (throughout development). Standard measures of cardiac function are well established in larger animals such as rabbits and dogs where invasive measurements can be made in anesthetized or chronically instrumented animals. Development of these same techniques in the mouse has only recently become available. In general this has necessitated the "miniaturization" of technology that allowes investigators reproducibly to assess cardiovascular function, both in the isolated perfused heart and in the intact anesthetized mouse.

The isolated Langendorff preparation and the working heart model have been elegantly characterized in several native mouse strains under varied physiologic conditions [35]. Several laboratories, including our own, have used the Langendorff model to provide direct evidence linking overexpression of heat shock protein with enhanced myocardial recovery following global ischemia in transgenic mouse lines [36, 37, 40].

Our laboratory has combined isolated heart perfusions with nuclear magnetic resonance (NMR) to study the effect of overexpression of heat-shock proteins in the heart on changes in pH and high-energy phosphates at rest and following global ischemia [40]. We have found that NMR spectroscopy, which is routinely used to study perfused hearts from larger species such as the rat, rabbit, and guinea pig, can be adapted to suit a 100-mg mouse heart. Figure 3 shows ^{23}Na-, ^{31}P-, and ^{13}C-NMR spectra obtained from three different perfused mouse heart protocols.

In panel A the ^{23}Na-NMR spectra have been obtained in the presence of TmDOTP, a shift-reagent which shifts the extracellular sodium (Na) signal away from the intracellular Na signal [66]. Shown in the upper left is a single spectrum in which the extracellular Na and intracellular Na resonance peaks are labeled. Spectra, shown in a stacked plot format, were acquired every 3 min at baseline, during 15 min of ischemia, and during 24 min of recovery. In this heart there is sustained elevation of intracellular Na into recovery.

In panel B the ^{31}P-NMR spectra were acquired every 5 min at baseline, during 15 min of ischemia, and then at 15 and 30 min of recovery. Shown are spectra at baseline, after 15 min of ischemia, and after 30 min of recovery. The phosphocreatine, inorganic phosphate, and three P resonances of adenosine triphosphate are labeled. Intracellular pH can be calculated from the chemical shift difference between the resonance peaks of phosphocreatine and inorganic phosphate [67]. These spectra show that there is incomplete recovery of adenosine triphosphate following this ischemic event.

In panel C there is a single ^{13}C-NMR spectrum of the C4 carbon of glutamate from a mouse heart extract. The extract was prepared after the isolated heart was subjected to 25 min of ischemia and then reperfused with ^{13}C-labeled acetate, octanoate (a short-chain fatty acid) and long-chain fatty acids. The resonance peaks marked with an asterisk arise from octanoate oxidation while the remainder arise from long-chain fatty acid oxidation. Analysis of this multiplet using ^{13}C-NMR isotopomer methods yields information about the relative contribution of octanoate and long-chain fatty acids to the acetyl coenzyme A pool from which glutamate is synthesized, in this case during reperfusion [68, 69]. This information may in turn provide insight into the postischemic activity of carnitine palmitoyl transferase (which is required for long-chain fatty acid metabolism).

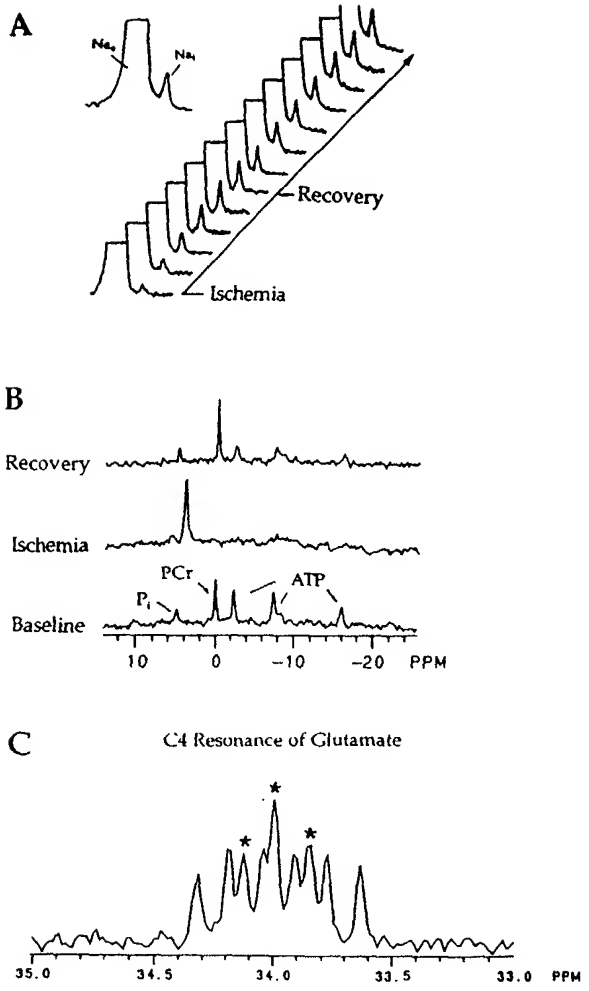

Fig. 3 A *Upper left*, a single ^{23}Na-NMR spectrum obtained in the presence of TmDOTP in which the extracellular Na and intracellular Na resonance peaks are labeled. *Right*, spectra, shown in a stacked plot format, were acquired at baseline, during 15 min of ischemia and during 24 min of recovery. B ^{31}P-NMR spectra acquired at baseline, after 15 min of ischemia and after 30 min of recovery. The phosphocreatine (*PCr*), inorganic phosphate (P_i) and three P resonances of adeosine triphosphate (*ATP*) are labeled. C ^{13}C-NMR spectrum of the C4 carbon of glutamate from a mouse heart extract which was prepared after the isolated heart was subjected to 25 min of ischemia and then reperfused with ^{13}C-labeled acetate, octanoate (a short-chain fatty acid), and long-chain fatty acids. The resonance peaks marked with an asterisk arise from octanoate oxidation while the remainder arise from long-chain fatty acid oxidation. Analysis of this multiplet using ^{13}C-NMR isotopomer methods yields information about the relative contribution of octanoate and long-chain fatty acids to the acetyl coenzyme A pool from which glutamate is synthesized

Optimally of course cardiovascular phenotypes would be interrogated in the intact animal. A number of invasive, catheter-based methods have been successfully applied to the mouse to make hemodynamic measurements in both the conscious and unconscious animal. Exciting advances have also been made in the application of non-invasive techniques such as magnetic resonance imaging and echocardiography to interrogate murine cardiac function.

The importance of the application of these methodologies to murine models of disease cannot be understated. Using a number of techniques outlined in Table 1, investigators have been able to characterize the cardiovascular phenotypes of a number of transgenic mice including lines which overexpress atrial natriuretic factor [25, 28], heat-shock proteins [36, 37, 40], human tissue kallikrein [27], β-adrenergic receptors [24], β-adrenergic receptor kinase and β-adrenergic receptor kinase inhibitor [23] as well as phospholamban deficient lines [32].

Summary

The advent of techniques to generate gain-of-function and loss-of-function mutations in laboratory animals represents one of the major accomplishments in cell and molecular biology in mammals over the past two decades. Although the technology is generally limited only to the mouse at present, substantial effort is underway to develop these techniques, and to refine existing techniques, in other species. Putative pluripotential ES cell lines have been derived in a number of other species including hamster [70], pig [71–75], sheep [73], cattle [76], rabbit [77], rat [78], mink [79], monkey [80], and even humans (81). Thus it seems likely the technology will be advanced into these additional species over the next few years, and each one of these may lend itself uniquely to problems ranging from development to tissue and organ physiology. Additionally, techniques such as those illustrated here and in Table 1 will need to be refined and applied to address each new mutation.

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Perspectives Series: Molecular Medicine in Genetically Engineered Animals

Transgenesis in the Rat and Larger Mammals

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Advances in biotechnology over the last ten years have made it possible for the researcher to alter gene expression in vivo in many diverse ways (1). With the establishment of embryonic stem (ES)[1] cell technology (2), more subtle and precise alterations can now be achieved than were previously possible using microinjection techniques. However, to date germline transmission has only been achieved with mouse ES cells, and microinjection continues to be the method most widely used for other species. While the mouse has a number of advantages, not least the depth of our knowledge of its genetics, other species are being increasingly used for transgenic studies due to their greater suitability for addressing specific questions. We will briefly review the application of transgenic technology to nonmurine species as it stands at present, with particular emphasis on developments appertaining to biomedical research.

Transgenesis by pronuclear injection

A number of significant limitations regarding the application of pronuclear injection to nonmurine animals have been identified (3), not least being the time and cost. Such limitations are due to longer gestation and generation times, reduced litter sizes, and higher maintenance costs. Further consideration must be given to the large numbers of fertilized eggs (and hence donor animals) required for microinjection, the high cost of carrying nontransgenic offspring to term, and the relatively low efficiency of gene integration. Such limitations are particularly severe for the production of bovine transgenics and, as a consequence, more significant departures from the standard procedures used for the mouse have been adopted for this species (4). For example, the use of in vitro embryo production in combination with gene transfer technology has played a large role in the development of transgenic cattle. The development of microinjected embryos through to the morula/blastocyst stage in recipient rabbits or sheep, enables sexing, transgene screening, and cloning to take place before reintroduction into the natural host, providing that such screening methods are robust and reliable.

The major problem regarding pronuclear microinjection is that the exogenous DNA integrates randomly into chromosomal DNA. Position effects, where the transgene is influenced by its site of integration in the host chromosome (5), can have major consequences on the expression of the transgene, including loss of cell specificity, inappropriately high copy number–independent expression and complete silencing of the transgene. This is of greater concern in nonmurine transgenesis where the investment is higher. Position-independent, copy number–related expression can be achieved using sequences such as the locus control regions identified upstream of the β-globin gene cluster and downstream of the CD2 gene (6, 7), the A elements which flank the chicken lysozyme gene (8), and matrix attachment regions (9). Such elements have been shown to function across species barriers, and their incorporation into gene constructs can overcome position effects and improve expression of heterologous genes within specific cell types (5). In many cases, simply including large amounts of flanking sequences may be sufficient to overcome position effects and direct expression to specific tissues. To this end, the development and use of P1 (10), bacterial artificial chromosome (BAC) (11) and yeast artificial chromosome (YAC) vectors (12) for cloning of large segments of DNA, should greatly improve the chances of including important regulatory elements, including those involved in chromatin structure, within the transgene construct.

Embryonic stem cell technology

With the development of ES cell technology in the mouse (2), genetic manipulations can be performed in cell culture using appropriate selection strategies to permit the directed integration of the transgene to a specific region of the chromosome via homologous recombination. With the advent of homologous recombination, the researcher is able to insertionally inactivate, replace, or introduce subtle alterations to the endogenous gene of interest. Once the intended genetic change has been verified, the appropriate ES cells are introduced into blastocysts by microinjection, and, during subsequent gestation, may contribute to the developing embryo. If such a contribution is made, then by definition the resulting animal would be chimeric, being derived in part from the ES cells originating in culture. Assuming that the chimerism extends to the germline, then an appropriate breeding strategy will lead to the recovery of nonchimeric heterozygotes and, if viable, mice which are homozygous for the genetic change.

Most attempts to isolate and culture inner cell mass (ICM) cells from other species are based on the methods used for the

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1. *Abbreviations used in this paper:* DAF, decay accelerating factor; ES, embryonic stem; HAR, hyperacute rejection; ICM, inner cell mass.

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mouse. ES cells are maintained in culture in the presence of mouse-derived differentiation-inhibiting agents, provided either as a media supplement or through cocultivation in the presence of feeder cells. It has been suggested that these mouse-derived agents do not adequately prevent differentiation of stem cells in species other than the mouse, and pluripotent rat ES cells, capable of producing chimeras, were found to grow best on primary rat embryonic fibroblasts as the feeder layer (13). Freshly isolated cells from ICMs have been injected into blastocysts to produce chimeric offspring in both sheep and cattle (14), and their totipotency at this stage is further demonstrated by their ability to produce offspring after transfer into enucleated oocytes (15). Such nuclear transfer techniques are potentially very useful for the production of clonal offspring and would avoid the initial chimeric generation necessitated by the injection of ES cells into blastocysts. Recently, bovine-specific culture methods have shown promise with cells of up to 27 d of age maintaining their ability to direct normal calf development following nuclear transfer (16). However, at the present time the reliable generation of bovine ES cell lines requires the pooling of ICMs from several blastocysts and further efforts are required to enable the long-term culture of clonal bovine ES cells. Although to date chimeric animals have been generated from several species including the pig (17), in no species other than the mouse has germline transmission of an ES cell been successfully demonstrated. This remains a major goal for the future and may well require the use of novel strategies which depart widely from the traditional methods used in the mouse.

Nonmurine species in biomedical research

Selected physiological questions may be more conveniently modelled in the rat or in larger species. Not only can physical size be an advantage for biochemical sampling and physiological analyses, but certain genes may provide useful information when introduced into, for example, the rat genome when parallel experiments in the mouse would be ineffective. Examples include the modulation of blood pressure by the mouse *Ren-2* gene (18) and the modeling of inflammatory disease (19). In both cases, but for different reasons, no phenotype was observed in the respective transgenic mice, highlighting one of the advantages of having alternative species for understanding physiological mechanisms and the etiology of disease. More recently, a number of transgenic experiments have been undertaken to investigate lipoprotein metabolism. The human apolipoprotein A-1 gene was successfully expressed in the rat (20), resulting in increased serum HDL cholesterol concentrations, and attempts to therapeutically lower apo B100, and hence LDL and lipoprotein(a) concentrations, in the rabbit were successful (21) but resulted in complications. Although the targeted expression of the apo B–editing protein in the liver of the transgenic rabbits resulted in reduced LDL and lipoprotein(a) concentrations as intended, many of the animals developed liver dysplasia, suggesting that high level expression of the editing protein had unforeseen and detrimental side effects, possibly via the editing of other important mRNAs. The rabbit has also been used in HIV-1 research, with the development of a line expressing the human CD4 protein on T lymphocytes (22). Susceptibility to HIV infection was demonstrated, and although the rabbits are less sensitive to infection than humans, they may represent an inexpensive alternative to primates for many studies.

Gene transfer in farm animals was initially aimed towards improving production efficiency, carcass quality (23), and disease resistance of livestock. However, it has been suggested that the simple over-expression of hormones such as growth hormone may have unacceptable side effects. Recently some elegant studies of growth using transgenic rats have been performed and are likely to yield valuable information on the biochemistry and physiology of growth (24, 25). A more successful application of transgenesis in farm animals has been the production of biomedically important proteins. The two most popular methods have been to direct expression to hematopoietic cells or to the lactating mammary gland. In the former case, transgenic swine expressing high levels of human hemoglobin were generated using the locus control region from the β-globin gene cluster to overcome positional effects and direct expression to the hematopoietic cells (26). However, due to its natural ability to synthesize and secrete large amounts of protein, the mammary gland has become the primary focus for the expression of heterologous proteins in large mammals. Transgene expression has been successfully directed to the mammary gland using promoter sequences from milk protein genes such as those encoding ovine β-lactoglobulin (BLG), goat β-casein, and murine whey acidic protein. The BLG promoter was used to direct expression of human α_1-antitrypsin in lines of transgenic mice and sheep (27). Interestingly, a wide variation in expression was observed between mouse lines, and from one lactation to another within a single line. In sheep however, similar high levels of heterologous protein were expressed in milk over consecutive lactations and over several generations in a given transgenic line, allowing the viable development of a flock of transgenic sheep. In separate studies high levels of expression of human tissue plasminogen activator were obtained in goat's milk under the control of the goat β-casein promoter (28). The development of suitable purification methods and the use of transgenically produced proteins in clinical trials are well advanced, and, if successful, will have important implications for the production of human proteins in transgenic livestock. Poor expression of the ovine promoter in the mouse may reflect species differences in recognizing heterologous versus homologous promoters and raises questions concerning the predictive value of mouse models. At best therefore the generation of transgenic mice may, in certain cases, only be a guide to the potential success of a transgene construct in another species.

Gene transfer could equally be used to enhance the quality and suitability of milk derived from domesticated animals as a food for human consumption. Human milk is devoid of β-lactoglobulin, which is responsible for most of the allergies to cows' milk, and has a relatively high content of lactoferrin, which is important in iron transport and combating bacterial infections. One could envisage in the future the reduction of saturated fat content in cows' milk and the knock-out of unwanted proteins or their replacement with other more useful components. Through the manipulation of milk constituents it should be possible to more closely emulate the desirable components of human milk. The alteration of milk composition would appear to be a practical possibility given that milk micelles are remarkably tolerant to changes in composition, as demonstrated by the knock-out of the mouse β-casein gene (29). Ethical concerns regarding the generation of transgenic animals, which have been engineered specifically for pharmaceutical, medical, or nutritional reasons, lie outside the scope

of this overview, however it must be clearly ascertained that expression of a transgene does not compromise the animal.

Xenograft organs for transplantation surgery

The shortage of human organs for transplantation has raised interest in the possibility of xenotransplantation, i.e. the use of animal organs (30). However, the major barrier to successful xenogeneic organ transplantation is the phenomenon of complement-mediated hyperacute rejection (HAR), brought about by high levels of circulating natural antibodies that recognize carbohydrate determinants on the surface of xenogeneic cells. After transplantation of the donor organ, a massive inflammatory response ensues through activation of the classical complement cascade. This leads to activation and destruction of the vascular endothelial cells and, ultimately, the donor organ. The membrane-associated complement inhibitors, endogenous to the donor organ, are species restricted and thus confer only limited resistance. The complement cascade is regulated at specific points by proteins such as decay accelerating factor (DAF), membrane cofactor protein, and CD59. These regulators of complement activation are species specific. The initial strategy used to address HAR in porcine-to-primate xenotransplantation was to produce transgenic pigs expressing high levels of the human terminal complement inhibitor, hCD59. This was shown to protect the xenogeneic cells from human complement-mediated lysis in vitro (31). More recently, organ transplantation has been achieved using donor pigs which expressed human DAF on their endothelium (32), or both DAF and CD59 on erythrocytes, such that the proteins translocated to the cell membranes of endothelial cells (33). After transplantation, the pig hearts survived in recipient baboons for prolonged periods without rejection (33). Clearly, such genetic manipulations are bringing xenotransplantation ever closer to reality. If the isolation of suitable ES cells and application of homologous recombination becomes a reality in the pig, it may be possible to knockout the antigenic determinants to which antispecies antibodies bind, as a further strategy for eliminating HAR.

Summary

The use of nonmurine species for transgenesis will continue to reflect the suitability of a particular species for the specific questions being addressed, bearing in mind that a given construct may react very differently from one species to another. The application of transgenesis in the pig should produce major advances in the fields of transfusion and transplantation technology, while alterations in the composition of milk in a range of domesticated animals will have major effects on the production of pharmacologically important proteins and could eventually lead to the development of human milk substitutes. Despite the lack of germline transmission to date, major efforts continue to be directed towards the generation and use of ES cells from nonmurine species, using both traditional and new technologies, and the availability of such cells is likely to accelerate both the use of such species and the precision with which genetic changes can be introduced.

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COMMENTARY

Human embryonic stem cells

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SUMMARY

Embryonic stem (ES) cells are cells derived from the early embryo that can be propagated indefinitely in the primitive undifferentiated state while remaining pluripotent; they share these properties with embryonic germ (EG) cells. Candidate ES and EG cell lines from the human blastocyst and embryonic gonad can differentiate into multiple types of somatic cell. The phenotype of the blastocyst-derived cell lines is very similar to that of monkey ES cells and pluripotent human embryonal carcinoma cells, but differs from that of mouse ES cells or the human germ-cell-derived stem cells. Although our understanding of the control of growth and differentiation of human ES cells is quite limited, it is clear that the development of these cell lines will have a widespread impact on biomedical research.

Key words: Human embryonic stem cell, Human embryo, Blastocyst, Primordial germ cell, Embryonal carcinoma, Mouse ES cell, Mouse EG cell, Marker, Growth regulation, Gene expression

INTRODUCTION

In November of last year, groups in the United States led by James Thomson and John Gearhart published data describing the derivation of candidate human pluripotent embryonic stem (ES) and embryonic germ (EG) cell lines from blastocysts or primordial germ cells, respectively (Thomson et al., 1998; Shamblott et al., 1998). Readers will probably agree that few if any previous scientific papers reporting the characterisation of cultured cell lines would have attracted a similar degree of public attention. The interest stems in part from the ethical controversy surrounding the origins of the cells but chiefly from the widespread conviction that their availability will profoundly alter our approaches to many problems in human biology and medicine. Several features define ES cells (below), but the two key properties that make these cells so remarkable are these: ES cells can be grown in vitro and expanded in number indefinitely in the primitive undifferentiated state characteristic of the embryonic cells from which they are derived, and throughout long periods of cultivation in vitro they retain a key property of those embryonic cells – pluripotency, or the ability to develop into any cell type in the adult body (Fig. 1). The scope of even the more obvious applications envisioned for human cells with these properties is breathtaking: new approaches to the study of human embryonic development and disorders thereof, such as birth defects and embryonal tumours; access to hitherto-unexplored territories of human embryonic gene expression for modern genomics data mining; new tools for the discovery of polypeptide growth and differentiation factors that might find application in tissue regeneration and repair; new means to creating human disease models in vitro for basic research, drug discovery and toxicology; a potential answer to the issue of the chronic shortage of tissue for transplantation in the treatment of degenerative diseases, and an end to the use of immunosuppressive therapy in transplantation, if cloning techniques can be used to derive stem cells from a patient's own tissue; new delivery systems for gene therapy.

Given the potential applications of these cells, and the ethical controversy regarding the use of in vitro fertilised embryos or tissue from aborted foetuses to derive them, the widespread public discussion of these issues is understandable, warranted, and welcome. However, since the sheer volume of commentary on human ES cell ethics, scientific applications and commercial potential now threatens to overwhelm the peer-reviewed scientific literature on the subject, we will focus here on human ES cells themselves: the background to their discovery, their known properties, and what we need to learn about them before we begin to use them to address the futuristic agenda outlined above.

PLURIPOTENT STEM CELLS IN MAMMALS

A brief historical account

The development of mouse ES cells in 1981 (Evans and Kaufman, 1981; Martin, 1981) provided the paradigm and as we will see below, much of the technology, for the development of human ES cells, but the concept of a pluripotent embryonic cell is far older than that. Development of ES cells evolved out

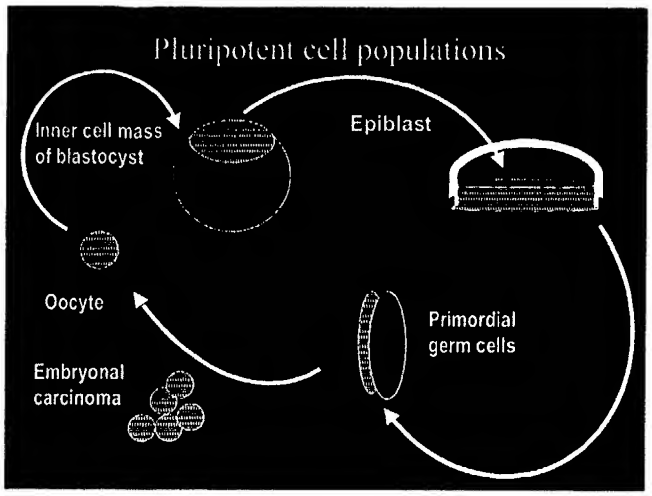

Fig. 1. Pluripotent stem cells. The oocyte, the cells of the early embryo up to compaction, the epiblast, primordial germ cells and the stem cells of teratocarcinomas all are pluripotent. Epiblast cells, primordial germ cells, and embryonal carcinoma cells can all give rise to cultured pluripotent stem cell lines.

of work on mouse teratocarcinomas, tumours that arise in the gonads of a few inbred strains, and consist of a remarkable array of somatic tissues juxtaposed together in a disorganised fashion. Classical work on teratocarcinomas established their origins from germ cells in mice and provided the concept of a stem cell (the embryonal carcinoma or EC cell) that can give rise to the multiple types of tissue found in the tumours (Kleinsmith and Pierce, 1964; review, Stevens, 1983). The field of teratocarcinoma research (review, Martin, 1980) expanded considerably in the 70's: the remarkable developmental capacity of the EC stem cell became apparent following the generation of chimaeric mice by blastocyst injection of EC cells, and investigators began to realise the potential value of cultured cell lines from the tumours as models for mammalian development. EC cells however had limitations: they often contained chromosomal abnormalities, and their ability to differentiate into multiple tissue types was often limited.

Since teratocarcinomas can also be induced by grafting blastocysts to ectopic sites, it was reasoned that it might be possible to derive pluripotent cell lines directly from blastocysts rather than from tumours, and that is what was done in 1981 by Gail Martin and Martin Evans independently. The result was a stable diploid cell line that could generate every tissue of the adult body, including germ cells. Teratocarcinomas also develop spontaneously from primordial germ cells in some mouse strains, or following transplantation of primordial germ cells to ectopic sites. In 1992 Brigid Hogan and her colleagues reported the direct derivation of EG cells from mouse primordial germ cells (Matsui et al., 1992). These EG cells have a developmental capacity very similar to that of ES cells, though they differ in their expression of some imprinted genes.

Testicular teratocarcinomas occur spontaneously in humans, and pluripotent cell lines were also developed from these (review, Andrews, 1988). Two groups reported the derivation of cloned cell lines from human teratocarcinoma that can differentiate in vitro into neurons and other cell types (Andrews et al., 1984; Thompson et al., 1984). Subsequently, cell lines were developed that can differentiate into tissues representative of all three embryonic germ layers (Pera et al., 1989). As analysis of the properties of human EC cells proceeded, it became clear that they are always aneuploid, usually (though not always) have a limited capacity for spontaneous differentiation into somatic tissue, and differ in phenotype from mouse ES or EC cells.

In 1995, James Thomson's team derived primate ES cells from rhesus monkey blastocysts and later from those of the marmoset (Thomson et al., 1995, 1996). These primate cell lines are diploid, and give rise to an extensive variety of tissues representative of all three embryonic germ layers, but otherwise closely resembled their nearest counterpart, the human EC cell. The implication of the monkey work and the work on human EC cells was that a pluripotent stem cell, which would be rather different in phenotype from a mouse ES cell, could probably be derived from a human blastocyst.

A generic functional definition of an ES cell

In considering the properties of ES or EG cells, there are certain generic features that any ES cell might be expected to possess, and other properties which may be peculiar to bona fide pluripotent cells isolated from different species or different tissues, or representative of a different stage of embryonic development. The mouse ES cell provides a benchmark for definition of the generic requirements for ES cells. Its key features are these: it is derived from a pluripotent cell population; it is stably diploid and karyotypically normal in vitro; it can be propagated indefinitely in the primitive embryonic state; it can differentiate spontaneously into multiple cell types representative of all three embryonic germ layers, both in teratomas after grafting or in vitro under appropriate conditions; and it can give rise to any cell type in the body, including germ cells, when allowed to colonise a host blastocyst. The criteria for pluripotency usually include derivation of the stem cell line from a single cloned cell. This experiment eliminates the possibility that several distinct committed multipotential cell types are present in the culture that together account for the variety of differentiated derivatives produced.)

Generic criteria for pluripotent ES or EG cells

- Originate from a pluripotent cell population
- Maintain normal karyotype
- Immortal and can be propagated indefinitely in the embryonic state
- Clonally derived cultures capable of spontaneous differentiation into extraembryonic tissue and somatic cells representative of all three embryonic germ layers in teratomas or in vitro

Thus far, only mouse EG or ES cells meet these generic criteria. Primate ES cells meet the first three four criteria, but not the last. Numerous other candidate mammalian ES cells have been described over the years in domestic and laboratory species, but only in the mouse have all criteria been met rigorously. Some workers restrict the designation of ES cell to those cells capable of colonising all tissues including the germ line. Such a test can never be applied to human ES cells for

ethical reasons. One can therefore refer to human cells as ES cells if they meet all other generic criteria, but should note that it may be very difficult to prove from in vitro data and teratoma formation in vivo that an ES cell really can give rise to every type of tissue cell, given that some tissue types are seen rarely, if ever, in teratomas or in vitro. Here, we will use the following terminology: a pluripotent stem cell is a stem cell derived from an embryo, primordial germ cells or a teratocarcinoma that is capable of indefinite self renewal and differentiation into extraembryonic tissue and tissue representative of all three embryonic germ layers; an ES cell is a preimplantation embryo-derived cell that meets the first four criteria listed above; and EG cell is a cell derived from the embryonic gonad that meets the first four criteria listed above.

THE HUMAN CELLS

Derivation and evidence for pluripotency

James Thomson and co-workers (1998) derived ES cells from surplus blastocysts donated by couples undergoing treatment for infertility. It is remarkable that their methodology was not very different from that used 17 years earlier to derive mouse ES stem cells: the trophectoderm, thought to be inhibitory to ES cell establishment, was removed by immunosurgery, the inner cell mass was plated on to a mouse embryonic fibroblast feeder cell layer, and following a brief period of attachment and expansion, the resulting outgrowth was disaggregated and replated onto another feeder cell layer. There were no significant departures from mouse ES protocols in the media or other aspects of the culture system and a relatively high success rate was achieved. Given that others have attempted to grow human ES cells, what factors were critical for success? The experience of Thomson's group with monkey ES cells was undoubtedly helpful. Primate pluripotent stem cells are different in many respects to mouse ES cells, particularly in their morphology and their ability to withstand dissociation into single cells, so it is important to recognise the correct cell type and handle it appropriately during subculture. But another factor that must be highlighted is the improvement in human embryo culture procedures, including the development of two stage culture systems that employ different media for appropriate developmental stages; this has allowed a high rate of production of good quality blastocysts (review, Gardner, 1998).

Shamblott and colleagues (1998) isolated pluripotent cells from embryonic and foetal gonads at 5-9 weeks post-fertilisation. Although we know little about the details of primordial germ cell maturation in man, we do know that this period encompasses those developmental stages in which primordial germ cells arrive in the gonads and proliferate, and overt sexual differentiation of the gonads occurs. Cells expressing markers characteristic of primordial germ cells are found in the human embryonic and foetal gonad throughout this period (Jorgensen et al., 1995). The culture system used by this group incorporated factors known to support mouse primordial germ cell survival and mitogenesis in vitro: STO fibroblast feeder cell layers, basic fibroblast growth factor, Leukemia Inhibitory Factor (LIF), and forskolin (review, Donovan, 1994).

To what extent do the cells described by both groups meet the generic criteria for ES or EG cells? Both types of culture are derived from pluripotent cell populations, and both retain a normal karyotype during extensive cultivation in vitro. The cells described by Thomson et al. have been grown for extensive periods and possess telomerase activity, both findings which indicate that they are immortal. The EG cells have not been grown for such long periods, but there is no indication that their lifespan is finite. The blastocyst-derived cells form teratomas containing derivatives of all three germ layers, and in some cases the tissues show a high degree of histotypic organisation (formation of ganglia, for example). Evidence for in vitro differentiation, however, is limited to expression of markers characteristic of trophoblast and endoderm formation (production of human chorionic gonadotrophin and alphafoetoprotein); whether the cells found producing alphafetoprotein represent extraembryonic (yolk sac) endoderm or definitive (embryonic) endoderm is not clear. In the case of the germ-cell-derived cultures, no evidence was presented regarding formation of teratomas in vivo, but the authors did observe in vitro differentiation within embryoid bodies. Embryoid bodies are structures formed by pluripotent stem cells grown in three-dimensional culture under conditions non-permissive for stem cell growth. In the mouse, embryoid bodies consist of two layers, one of extraembryonic endoderm and one of ectoderm, and interactions between these two cell types probably drive the differentiation of ectoderm into multiple cell lineages, which mimics the situation in the early postimplantation embryo in vivo (review, Martin, 1981). Shamblott et al. sectioned embryoid bodies that formed spontaneously in culture and, using immunochemistry, demonstrated expression of single markers in different cell types consistent with the representation of mesodermal, ectodermal and endodermal lineages. It may be premature to refer to these structures as embryoid bodies, given that as yet no convincing evidence based on marker or gene expression shows that they resemble any structure found in the human peri-implantation embryo. However, they clearly contain a heterogenous mixture of cell types. Neither group was able to clonally derive cell lines, although this might be only a matter of persistence. Our own studies on ES cell lines independently derived from blastocysts confirm the findings of J. A. Thomson et al. (B. E. Reubinoff, M. F. Pera, C. Y. Fong, A. O. Trounson and A. Bongso, unpublished).

In summary the evidence that ES cells can be derived from human blastocysts is quite convincing, although stronger data regarding somatic differentiation in vitro and clonal derivation would prove this conclusively. The evidence for pluripotency of the EG cells is also strongly suggestive, although additional data on teratoma formation, the characterisation of the differentiated cells found in vitro, and clonal derivation would make the case more convincing.

Morphology, marker expression and growth requirements

How do the phenotypes of the cells – morphology, antigen expression, growth requirements – compare with one another and with those of other types of pluripotent cells, such as EC cells or mouse ES cells? Human EC cells and monkey and human ES cells have phenotypes which are very similar and readily distinguished from those of their counterparts in the mouse and from those of human EG cells. The primate cells

Table 1. Marker expression and growth properties of mouse and primate pluripotent cells

	Mouse EC, ES, EG cells	Human EC cells	Monkey ES cells	Human ES cells	Human EG cells
SSEA-1	+	–	–	–	+
SSEA-3	–	+	+	+	+
SSEA-4	–	+	+	+	+
TRA-1-60	–*	+	+	+	+
TRA-1-81	–*	+	+	+	+
GCTM-2	–*	+	+‡	+	?
Alkaline phosphatase	+	+	+	+	+
Oct-4	+	+	+‡	?	?
Genesis	+ (ES)	+	?	?	?
Germ cell nuclear factor	+ (ES, EC)	+	?	?	?
GDF-3	+ (ES, EC)	+	?	?	?
Cripto (TDGF-1)	+ (inner cell mass, ectoderm)	+	?	?	?
Feeder-cell dependent	ES, EG, some EC	Some; few show high cloning efficiency	Yes	Yes	Yes
Factors known to aid stem cell renewal	LIF and other factors acting through gp130	?	?	?	LIF, bFGF

*Antibodies do not react with mouse cells. It is unknown whether this is due to lack of expression or species specificity of the antibody.
‡B. E. Reubinoff and M. F. Pera (unpublished).

grow in flat colonies with distinct cell borders in monolayer culture whereas mouse ES cells grow in more rounded clumps with indistinct cell borders. A series of surface antigens characterise primate pluripotent stem cells. The stage-specific embryonic antigens 1, 3 and 4 are globoseries glycolipids recognised by monoclonal antibodies originally raised to distinguish early stages of mouse development. Primate pluripotent cells express SSEA-3 and SSEA-4 (the epitope recognised by the latter is more readily detected than that seen by the former), and express SSEA-1 only upon differentiation (Andrews et al., 1996; Thomson and Marshall, 1998; Thomson et al., 1998). Essentially the reverse is true of mouse ES cells. Also characteristic of human EC cells is the expression of a set of antigens associated with a pericellular matrix proteoglycan found on the surface of these cells that is also secreted or shed into the culture medium by them (Cooper et al., 1992; Badcock et al., in press). The TRA-1-60 epitope is a sialidase-sensitive epitope associated with this proteoglcyan; the antibody GCTM-2 reacts with its core protein, and antibodies TRA-1-80 and K21 react with other unknown epitopes on the same molecule. Human ES cells, as well as monkey ES cells, react with TRA1-60, TRA1-80 and GCTM-2. Although GCTM-2 and TRA1-60 do not label mouse ES or EC cells, it is not clear whether the mouse cells lack the surface proteoglycan or whether the antibodies are species specific. Peter Andrews and co-workers (Badcock et al., 1999) have pointed out that mouse ES and EC cells, and their human counterparts, all express some form of polylactosamine glycoconjugate on their surface, whether it be keratan sulphate or a non-sulphated form. All primate pluripotent stem cells, like mouse EC and ES cells, express alkaline phosphatase activity. In humans, there are four different isozymes of alkaline phosphatase. EC cells express the tissue non-specific form and a form of the enzyme that can be detected by antibodies that react with the germ cell or placental form (available immunological reagents do not distinguish between these closely related isoforms). It is not clear which form of alkaline phosphatase the human ES cells express.

It must be noted that none of these surface markers is completely specific and all can be detected in other tissue types. The markers analysed thus far in the primate or human ES or EG cells are only immunochemically defined epitopes or enzymatic activities; there is as yet little information on gene expression in human ES cells or primate ES cells.

Like mouse ES and EG cells, primate pluripotent cells, including some human EC cells, require a mouse embryonic fibroblast feeder-cell layer for support. (The terminology used for these feeder cells is misleading. They are better described as primitive mesenchymal cells rather than connective tissue fibroblasts, and they are derived from midgestation foetuses, not embryos.) In the case of mouse ES and EG cells, this requirement can be replaced by LIF or related members of this cytokine family, but pluripotent human EC cells, rhesus monkey ES cells, and human ES cells will not respond to LIF in such a fashion (Pera et al., 1989; Roach et al., 1993; Thomson and Marshall, 1998; Thomson et al., 1998). Even on a feeder cell layer, all primate pluripotent cells grow very poorly when dissociated to single cells, whereas mouse ES cell lines can be cloned at a relatively high efficiency in the presence of LIF under these conditions.

The morphology, marker expression and growth requirements of pluripotent cells derived from the gonad differ in some ways from those of other primate pluripotent stem cells. The cells grow in more rounded clumps that lack distinct cell borders and are very difficult to dissociate. They express SSEA-1 in addition to SSEA-3, SSEA-4, and TRA 1-60, and they contain alkaline phosphatase activity. The EG cells appear to exhibit some degree of dependence on LIF and basic fibroblast growth factor, although this has not yet been systematically investigated. It must be noted that the markers expressed by these germ cell derived cultures are consistent with the identification of these cells as primordial germ cells. As in the mouse, the process of conversion from a primordial germ cell to a cell that can be continuously cultured and that is pluripotent is poorly defined. Shamblott et al. noted that only a small fraction of the cells give rise to embryoid bodies containing multiple types of differentiated cell. This process of conversion to pluripotency may be slower in humans, since the time frame of germ cell maturation is different to that in the mouse.

Generic molecular markers of pluripotent stem cells?

Although there are apparent differences in the phenotype of murine and primate pluripotent cells, a universal set of molecular markers might be common to all types of stem cell. Such markers might not be absolutely specific to pluripotent cells but would probably have a very restricted expression in other cell types. The data on primate ES and EG cells are very limited at present, but a comparison of mouse ES and EC cells and human EC cells enables a preliminary sketch of the universal pluripotent mammalian stem cell to be drawn. Potential molecular markers include high molecular mass cell surface polylactosamine glycoconjugates, expression of some form of alkaline phosphatase, expression of the growth factors GDF-3 (Caricasole et al., 1998) and Cripto or Teratocarcinoma Derived Growth Factor 1 (Baldassare et al., 1997; Xu et al., 1998), and expression of the transcription factors OCT-4 (review, Brehm et al., 1998), germ cell nuclear factor (Lei et al., 1997) and Genesis (Sutton et al., 1996). The biological roles of most of these molecules in the context of ES cell growth remain to be defined, with the exception of Oct-4, which is required for the establishment of the pluripotent cell lineage in the mouse (Nichols et al., 1998).

CONCLUSIONS: HUMAN ES CELL PROSPECTIVES

Most of the applications of human ES cells will require cells to be grown and manipulated as a relatively pure stem cell population on a large scale, and the availability of methods for producing and isolating specific types of differentiated cell from them. At present, no one has reported large scale growth, efficient cloning or genetic manipulation of human ES or EG cells. It will be important to identify factors that facilitate growth and inhibit differentiation of human ES cells. Liberation from a feeder cell requirement may be essential for certain types of experiments, as well as for production of cells for transplantation. Despite many years of use of mouse embryonic fibroblast feeder cell layers for support of mouse ES cells and other cell types, it is not yet clear at the molecular level exactly what these cells provide for their clients. The feeder cell effect on primate pluripotent cells is not reproduced by either supernatants from the cells or by the extracellular matrix which they secrete onto the monolayer (Pera et al., 1989; Thomson and Marshall, 1998), which suggests either that secreted factors and matrix function synergistically, or that the important factors are either membrane bound, are passed through gap junctions, or are highly unstable. In the case of primate pluripotent cells, even in the presence of the mouse feeder cell layer, both their cloning efficiency and their tolerance for dissociation to single cells is very low. Therefore it seems likely that juxtacrine factors, again possibly membrane bound ligands binding to receptors on neighbouring autologous cells, are also critical to human ES cell growth or survival. There are several candidate positive regulators of ES cell growth, none of which has yet been purified to homogenity or cloned; these factors appear unrelated to LIF and might eventually be found to play an important role in the maintenance of human ES cells (Roach et al., 1993; Dani et al., 1998; Rathjen et al., 1999).

In the case of mouse ES cells, and probably in the case of human ES cells, certain types of differentiated cell inhibit continuous stem cell growth. A methodology called stem cell selection, in which a selectable marker under the control of a stem cell specific promoter such as Oct-4 is introduced into stem cells, enables selection against differentiated cells during routine subcultivation, facilitating removal of inhibitors and enhanced stem cell growth (McWhir et al., 1996). Modulation of the action of whatever inhibitors these differentiated cells produce should also promote stem cell proliferation.

Directed differentiation of human ES cells into specific lineages has not yet been achieved. Spontaneous differentiation of mouse ES, human EC, and human EG cells has been observed both in embryoid bodies and in high density cultures. In mouse ES and human EC systems, retinoic acid or polar solvents, or growth factors, induce differentiation into cell populations that are enriched for particular cell types. The range of cells observed in teratomas formed in xenografts of human ES or human EC cells usually exceeds that seen during routine cultivation in vitro. Therefore, either the induction and proliferation of committed progenitor cells depend upon environmental factors that are absent in vitro, or factors present in vitro block differentation into specific lineages. Alternatively, a combination of these two possibilities might act to limit the variety of cell types seen in culture. It is possible that simpler culture systems that eliminate the effects of feeder cells and components of serum will be necessary to enable a given purified factor or combination of factors to drive stem cells into commitment to a particular lineage (Wiles and Johansson, 1999). Alternatively, selective culture conditions or the use of lineage specific stem cell selection might allow isolation of pure populations of precursor cells appearing in a mixed background of cell types after spontaneous differentiation (Li et al., 1998).

There are clearly many challenges for cell biologists in this arena, and the opportunities are vast. The rapid dissemination of this technology, full support of this research from governmental and philanthropic as well as private sector sources, and cooperation and collaboration amongst workers in the area, will ensure that the potential benefits to research and medicine are realised soon.

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Targeted Gene Replacement

Researchers can now create mice bearing any chosen mutations in any known gene. The technology is revolutionizing the study of mammalian biology

by Mario R. Capecchi

Every cell of our bodies has within its nucleus an instruction manual that specifies its function. Although each cell carries the same manual, different cell types, such as liver or skin, use different parts of this manual to detail their unique functions. Perhaps most remarkable, the manual contains the information that allows a one-cell embryo, the fertilized egg, to become a fetus and then a newborn child. As the child matures physically and intellectually, he or she is still using the information within the instruction manual. We are each unique, and the manual is slightly different for each of us; it specifies most of the physical and many of the behavioral characteristics that distinguish us as individuals.

This extraordinary manual, otherwise known as the genome, is written in the form of nucleotides, four of which constitute the entire alphabet—adenylate (*A*), cytidylate (*C*), guanylate (*G*) and thymidylate (*T*). It is the precise sequence of the nucleotides in DNA that conveys information, much as the sequence of letters in a word conveys meaning. During each cell division, the entire manual is replicated, and a copy is handed down from the mother cell to each of its two daughters. In humans and mice, the manuals each contain three billion nucleotides. If the letters representing the nucleotides were written down in order so that a page carried 3,000 characters, the manual would occupy 1,000 volumes, each consisting of 1,000 pages. Thus, a very complex manual is required to orchestrate the creation of a human or mouse from a fertilized egg.

Recently my colleagues at the University of Utah and I developed the technology for specifically changing a letter, a sentence or several paragraphs in the instruction manual within every cell of a living mouse. By rewriting parts of the manual and evaluating the consequences of the altered instructions on the development or the postdevelopmental functioning of the mouse, we can gain insight into the program that governs these processes.

The functional units within the instruction manual are genes. We specifically change the nucleotide sequence of a chosen gene and thereby alter its function. For instance, if we suspected a particular gene were involved in brain development, we could generate mouse embryos in which the normal gene was "knocked out"—that is, completely inactivated. If this inactivation caused newborn mice to have a malformed cerebellum, we would know that the gene in question was essential to forming that part of the brain. The process by which specified changes are introduced into the nucleotide sequence of a chosen gene is termed gene targeting.

Much of what is learned from gene-targeting experiments in mice should benefit humans, because an estimated 99 percent or more of the genes in mice and humans are the same and serve quite similar purposes. Application of the technology in mice is already clarifying not only the steps by which human embryonic development occurs but also the ways in which our immune system is formed and used to fight infection. Gene targeting should also go far toward explaining such mysteries as how the human brain operates and how defects in genes give rise to disease. In the latter effort the technique is being used to produce mouse models of human disorders—among them, cystic fibrosis, cancer and atherosclerosis.

Excitement over gene targeting stems from another source as well. It promises to expand on the knowledge generated by the genome project. This large-scale undertaking aims to determine the nucleotide sequence of every gene in the mouse and human genomes (approximately 200,000 genes in each). Currently we know the functions of only a minute percentage of the genes in either species. The nucleotide sequence of a gene specifies the amino acids that must be strung together to make a particular protein. (Proteins carry out most of the activities in cells.) The amino acid sequence of a protein yields important clues to its roles in cells, such as whether it serves as an enzyme, a structural component of the cell or a signaling molecule. But the sequence alone is not sufficient to reveal the particular tasks performed by the protein during the life of the animal. In

TARGETED MUTATION can be generated in a selected cellular gene by inserting mutated copies of the gene (*green-and-gold strips at far left*) into cells and allowing one copy to take the place of the original, healthy gene (*gold fragment at far right*) on a chromosome. Such altered cells are helping researchers to produce mice carrying specific genetic mutations. The finding of a curled tail and a balance-and-hearing disorder in one such mouse (*above*) led to the discovery that the affected gene, *Int-2*, participates in development of the tail and the inner ear.

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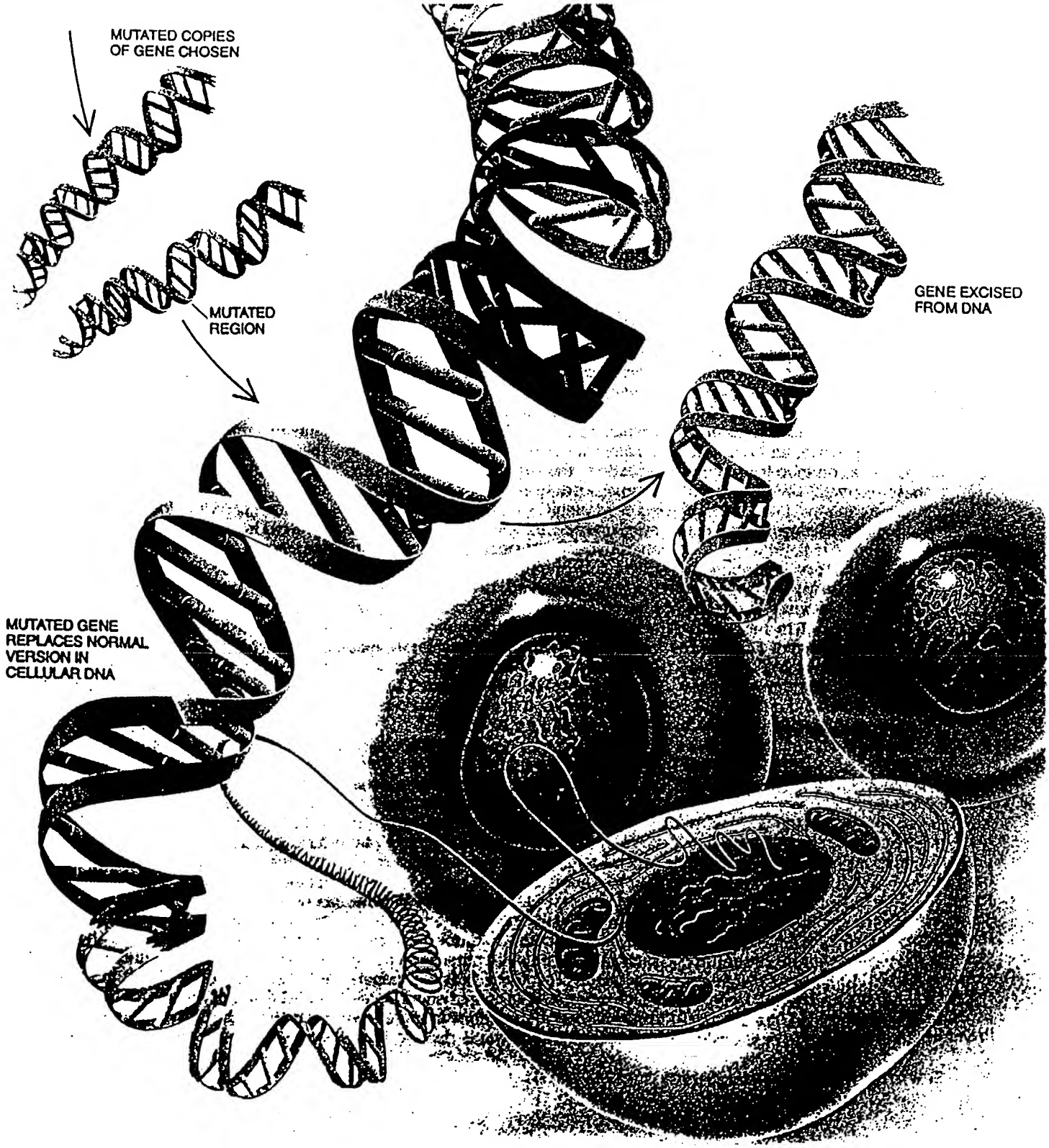
contrast, gene targeting can provide this information and thereby move our understanding of the functions of genes and their proteins to a much deeper level.

Gene targeting offers investigators a new way to do mammalian genetics—that is, to determine how genes mediate various biological processes. This technique was needed because the classical methods of genetics, which have been highly successful in analyzing biological processes in simpler organisms, were not readily adaptable to organisms as complex as mammals.

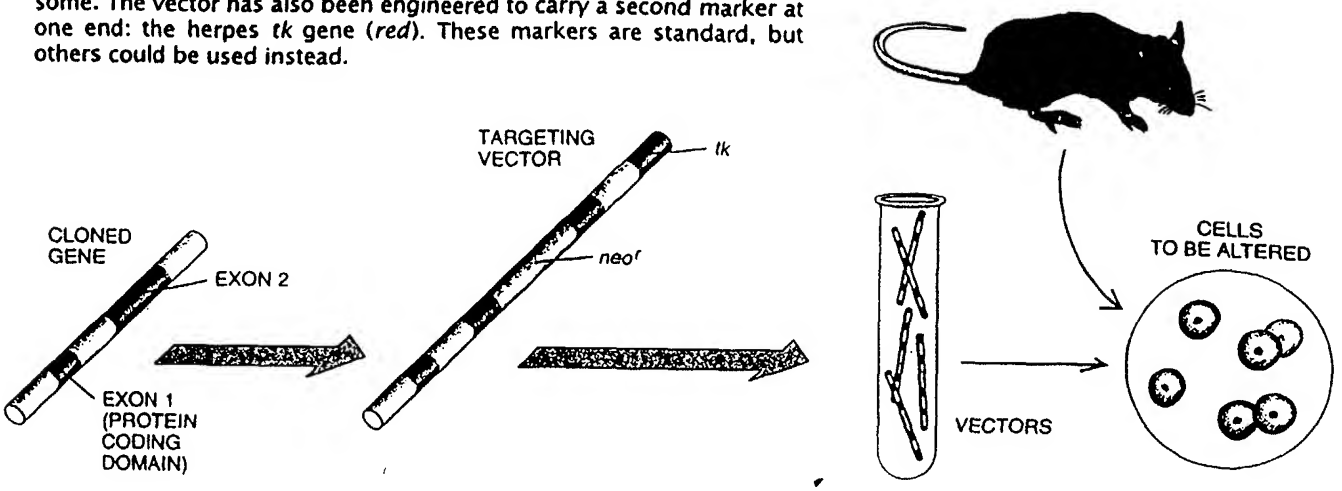
If geneticists want to learn, for example, how single-cell organisms, such as bacteria or yeast, replicate their DNA, they can expose a billion or more individuals to a DNA-damaging chemical (a mutagen). By choosing the right dosage of mutagen, they can ensure that each individual in that population carries a mutation in one or more genes. From this population of mutagenized bacteria or yeast, the geneticists can identify individuals not capable of replicating their DNA. The use of such a large mutagenized population makes it likely that separate individuals will be found with mutations in each of the genes required for DNA replication. (For a process as complicated as duplicating the bacterial or yeast genome, more than



How Targeted Gene Replacement Is Accomplished in Cultured Cells

1. Workers alter copies of a gene (*strip at far left*) in the test tube to produce what is called the targeting vector (*lengthened strip*). The gene shown here has been inactivated by insertion of the *neo*^{r} gene (*green*) into a protein coding region (*blue*). The *neo*^{r} gene will serve later as a marker to indicate that the vector DNA took up residence in a chromosome. The vector has also been engineered to carry a second marker at one end: the herpes *tk* gene (*red*). These markers are standard, but others could be used instead.

2. Once a vector, with its dual markers, is complete, it is introduced into cells (*gray*) isolated from a mouse embryo.



100 genes are involved.) Once the individual genes are identified, their specific role in DNA replication, such as which genes control the decision to copy the DNA and which control the accuracy and rate of copying, can be determined.

Similar approaches have been applied to multicellular organisms, which are more complex. Two favorites of geneticists are *Caenorhabditis elegans*, a tiny, soil-dwelling worm, and *Drosophila melanogaster*, a common fruit fly. But even in these relatively simple forms of multicellular organisms, identifying all the genes involved in a specific biological process is more demanding.

A number of factors contribute to this increased difficulty. One is the size of the genome. The genome of the bacterium *Escherichia coli* includes only 3,000 genes, whereas that of *D. melanogaster* contains at least 20,000 genes; the mouse genome contains 10 times that number. With added genes comes added complexity, because the genes form more intricate, interacting networks. Tracing the effect of any one gene in such an involved network is a formidable task.

Moreover, the larger size of multicellular organisms places practical limits on the number of individuals that can be included in a mutagenesis experiment. It is fairly simple and inexpensive to search for specific kinds of mutants among more than a billion mutagenized bacteria or yeast. In contrast, screening even 100,000 mutagenized fruit flies would constitute a large experiment. By comparison, the practical limits on screening mice for a particular mutation would be reached at about 1,000 animals.

The logistical difficulties of identifying and studying genes in multicellular organisms are further increased by the fact that most are diploid—their cells carry two copies of most genes, one inherited from the father and a second from the mother. For survival purposes, having two copies of most genes is valuable. If one copy acquires a harmful mutation, the other copy can usually compensate, so that no serious consequences result. Such redundancy, however, means that a mutation will elicit anatomical or physiological defects in the organism only if both copies of the gene are damaged. Investigators produce such individuals by mating parents who each carry the mutation in one copy of the gene. Approximately one fourth of the offspring of such matings will bear two defective copies of the gene. The need for matings introduces delays in the analysis.

Despite the challenges, the identification of selected mutations in whole animals is unquestionably the most informative way to begin clarifying and separating the steps by which biological processes are carried out. Furthermore, if we want to understand processes that occur only in complex organisms, such as the mounting of a sophisticated immune response, such analysis must be pursued in those organisms. For these reasons, geneticists interested in mammalian development, neural function, immune response, physiology and disease have turned to the mouse. From a geneticist's point of view, the mouse is an ideal mammal. It is small and prolific and serves as a remarkably good analogue for most human biological processes.

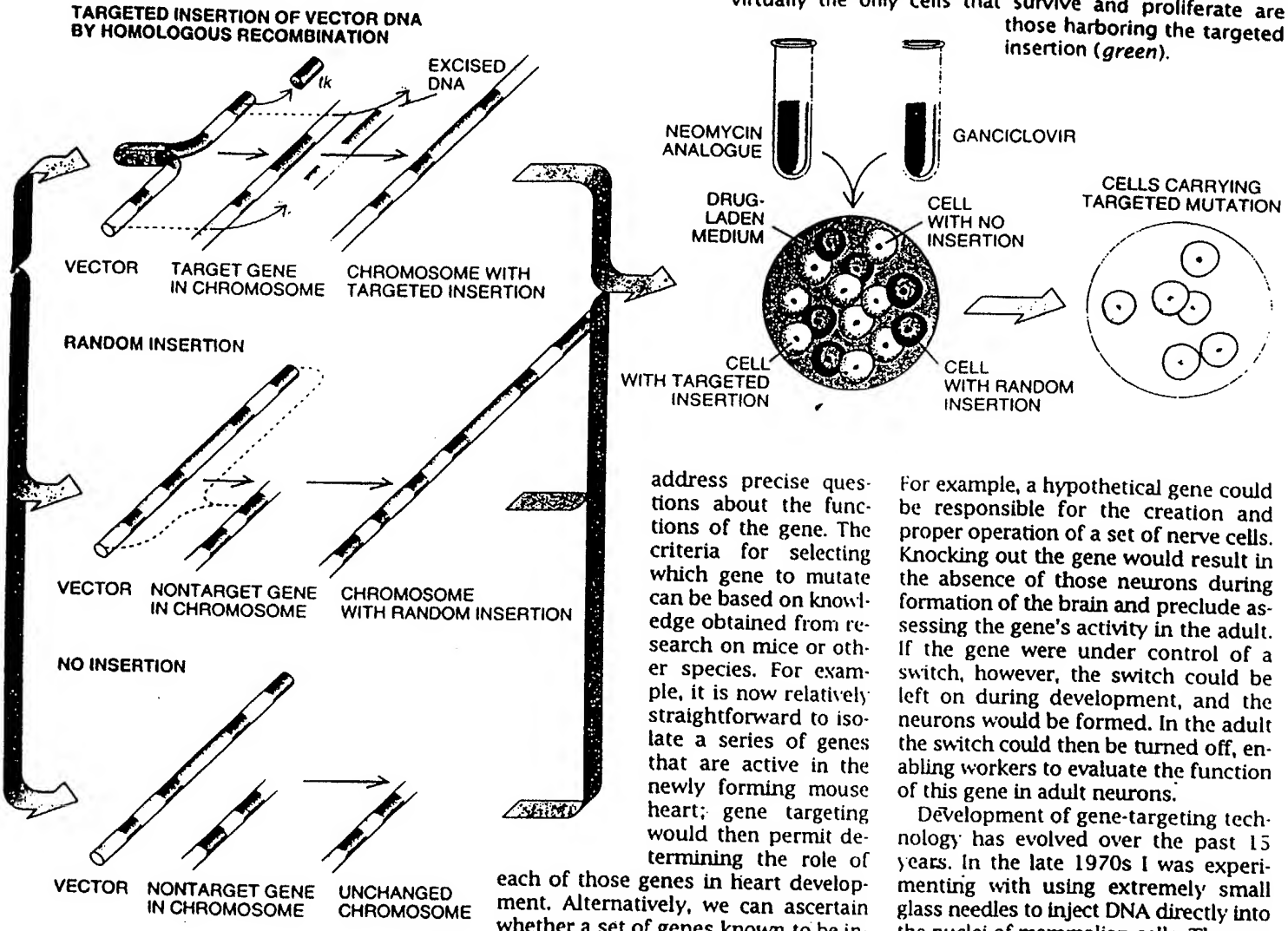
On the other hand, the breadth of genetic manipulations that can be carried out in mice has been extremely limited relative to the operations that are possible in simpler organisms. Because of the obstacles I have already described, it is not practical to apply classical techniques to mice. To identify mutagenized mice carrying defects in the genes involved in some process of interest, researchers would have to screen 10,000 to 100,000 mice at a prohibitive cost. Instead mouse geneticists have historically studied mutant animals that arose spontaneously within their colonies. As a result of the keen observation and perseverance by such workers, the collection of existing mouse mutants is surprisingly large and is an invaluable resource for continued research.

Yet even these hard-won animals have drawbacks. The existing collection of mutant mice does not harbor a random sampling of mutations in the mouse genome. Rather it contains a disproportionate number of mutations that result in readily observable abnormalities in physiology or behavior. In consequence, many mutations that affect coat color are present in this collection, whereas mutations that affect early development are underrepresented (since they often result in the undetected death of the embryo).

Further, the task of isolating the genes responsible for overt defects in

3. When all goes well, homologous recombination occurs (*top*): the vector lines up next to the normal gene (the target) on a chromosome in a cell, so that the identical regions are aligned; then those regions on the vector (together with any DNA in between) take the place of the original gene, excluding the marker at the tip (*red*). In many cells, though, the full vector (complete with the extra marker) fits itself randomly into a chromosome (*middle*) or does not become integrated at all (*bottom*).

4. To isolate cells carrying a targeted mutation, workers put all the cells into a medium containing selected drugs, here a neomycin analogue (G418) and ganciclovir. G418 is lethal to cells unless they carry a functional *neo*^{r} gene, and so it eliminates cells in which no integration of vector DNA has occurred (*gray*). Meanwhile ganciclovir kills any cells that harbor a *tk* gene, thereby eliminating cells bearing a randomly integrated vector (*red*). Consequently, virtually the only cells that survive and proliferate are those harboring the targeted insertion (*green*).



mutant mice is very labor intensive, often taking years of concerted effort. Workers can deduce many steps involved in biological phenomena without ever finding the genes involved. But without isolating those genes, they cannot make progress at the molecular level. Notably, they cannot determine the nature of the proteins encoded by the mutated genes, nor can they identify the cells in which the genes are active.

Gene targeting allows investigators to circumvent such difficulties. Investigators now choose which gene to alter. They also have virtually complete control over how that gene is modified, so that the mutation can be tailor-made to address precise questions about the functions of the gene. The criteria for selecting which gene to mutate can be based on knowledge obtained from research on mice or other species. For example, it is now relatively straightforward to isolate a series of genes that are active in the newly forming mouse heart; gene targeting would then permit determining the role of each of those genes in heart development. Alternatively, we can ascertain whether a set of genes known to be involved in guiding the paths taken by developing neurons in *D. melanogaster* exist and serve a similar function in the mouse.

An initial approach often involves knocking out a gene in order to evaluate the consequences to the organism of not having the gene product. The consequences may be complex and may affect multiple pathways. Further insight into the gene's function can be obtained by introducing more subtle, defined mutations, which may affect only one of its multiple roles. Soon geneticists should be able to place genes under control of a switch. Such switches will allow researchers to turn a gene on and off during the embryonic or postnatal development of the mouse. For example, a hypothetical gene could be responsible for the creation and proper operation of a set of nerve cells. Knocking out the gene would result in the absence of those neurons during formation of the brain and preclude assessing the gene's activity in the adult. If the gene were under control of a switch, however, the switch could be left on during development, and the neurons would be formed. In the adult the switch could then be turned off, enabling workers to evaluate the function of this gene in adult neurons.

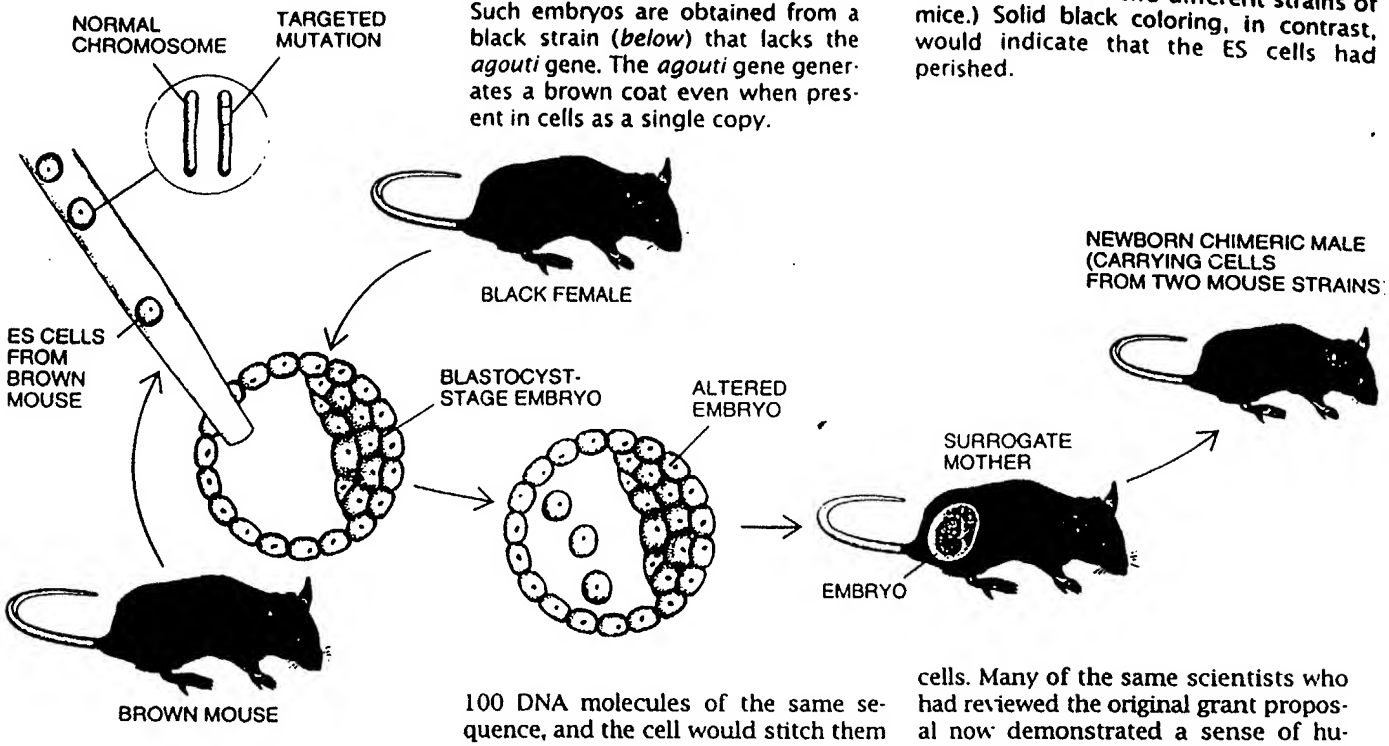
Development of gene-targeting technology has evolved over the past 15 years. In the late 1970s I was experimenting with using extremely small glass needles to inject DNA directly into the nuclei of mammalian cells. The needles were controlled by hydraulically driven micromanipulators and directed into nuclei with the aid of a high-powered microscope. The procedure turned out to be extremely efficient. One in three to five cells received the DNA in a functional form and went on to divide and stably pass that DNA on to its daughter cells.

When I followed the fate of these DNA molecules in cells, a surprising phenomenon captured my attention. Although the newly introduced DNA molecules were randomly inserted into one of the recipient cell's chromosomes, more than one molecule could be inserted at that site, and all of them were in the same orientation. Just as words in any language have an orienta-

How Targeted Gene Replacement Is Accomplished in Mice

1. Cells known as embryonic stem (ES) cells (*green at far left*) are isolated from a brown mouse strain and altered (by the process described in the illustration on pages 36 and 37) to carry a targeted mutation in one chromosome (*inset*). The ES cells are then inserted into young embryos, one of which is shown. Workers like to use the coat color of the future newborns as a guide to whether the ES cells have survived in the embryo. Hence, they typically put the ES cells into embryos that, in the absence of the ES cells, would acquire a totally black coat. Such embryos are obtained from a black strain (*below*) that lacks the *agouti* gene. The *agouti* gene generates a brown coat even when present in cells as a single copy.

2. The embryos containing the ES cells grow to term in surrogate mothers. Then workers examine the coats of the newborns. Brown shading intermixed with black indicates that the ES cells have survived and proliferated in an animal. (Such individuals are called chimeras because they contain cells derived from two different strains of mice.) Solid black coloring, in contrast, would indicate that the ES cells had perished.



tion (in English we read words from left to right), so, too, do DNA molecules. Apparently, before cells performed random insertion, some mechanism in the cell nucleus stitched virtually all the introduced DNA molecules together in the same orientation.

We went on to demonstrate that cells used a process called homologous recombination to achieve such linkages. Homologous recombination works only on DNA molecules with the same nucleotide sequence. Such molecules line up next to each other. Then both molecules are cut and are joined to each other at the cut ends. The joining is accomplished with such precision that the nucleotide sequences at the points of linkage are not altered.

This unexpected observation implied that all mouse cells, and presumably all mammalian cells, had the machinery to perform homologous recombination. At the time, there was no reason to suspect that somatic cells (those not involved in sexual reproduction) would have this machinery. Further, we knew the machinery was fairly efficient because we could microinject more than 100 DNA molecules of the same sequence, and the cell would stitch them all together in the same orientation. I realized immediately that if we could harness this machinery to carry out homologous recombination between a newly introduced DNA molecule of our choice and the same DNA sequence in a cell's chromosome, we would have the ability to rewrite the cell's instruction manual at will.

Excited by this prospect, in 1980 I requested funding from the government to test the feasibility of gene targeting. To my disappointment, the scientists who reviewed the grant proposal rejected it. In their view, the probability that the newly introduced DNA sequence would ever find its matching sequence within the 1,000 volumes of the genetic instruction manual seemed vanishingly small.

Despite the rejection, I decided to forge ahead using funds I was receiving for another project. It was a gamble. Had the experiments failed, I would have had little meaningful data to submit at grant renewal time. Fortunately, the experiments worked. By 1984, when we again asked for funds to pursue the research, we had ample evidence that gene targeting was in fact feasible in cells. Many of the same scientists who had reviewed the original grant proposal now demonstrated a sense of humor. The critique of the new proposal opened with the statement, "We are glad that you didn't follow our advice."

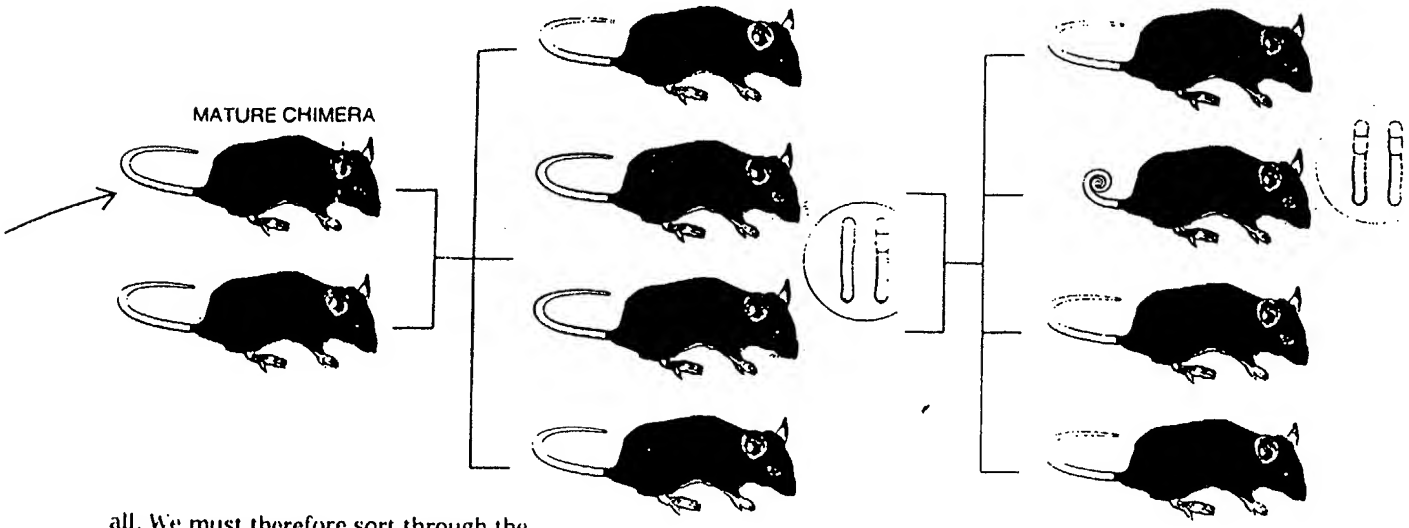
How is gene targeting in cells accomplished? The first step is to clone the gene of interest and propagate it in bacteria. This procedure provides a pure source of DNA containing the gene. Next, in a test tube, the nucleotide sequence of the gene is changed to meet the purpose of the experiment. The altered gene is referred to as the targeting vector.

The targeting vector is introduced into living cells by any of several means. Once within the cell nucleus, it forms a complex with proteins constituting the cell's machinery for homologous recombination. Aided by these proteins, it searches through all the sequences of the genome until it finds its counterpart (the target). If it indeed does find its target, it will line up next to that gene and replace it.

Regrettably, such targeted replacement occurs only in a small fraction of the treated cells. More often, the targeting vector inserts randomly at nonmatching sites or fails to integrate at

3. Chimeric males are mated to black (non-*agouti*) females. Then researchers screen the progeny for evidence of the targeted mutation (*green in inset*) in the gene of interest. They exclude black mice immediately; if the animals had been born of sperm made by ES cells—and so had a chance of harboring the chosen mutation—they would be brown. Direct examination of the genes in the brown mice reveals which of those animals (*boxed*) inherited the targeted mutation.

4. Males and females carrying the mutation are mated to each other to produce mice whose cells carry the chosen mutation in both copies of the target gene (*inset*) and thus lack a functional gene. Such animals (*boxed*) are identified definitively by direct analyses of their DNA. Then they are examined carefully for any physical or behavioral abnormalities.



all. We must therefore sort through the cells to identify those in which targeting has succeeded. Approximately one in a million treated cells has the desired targeted replacement.

To greatly simplify the search for that cell, we make use of two "selectable markers," which are introduced into the targeting vector from the start. Inclusion of a "positive" selectable marker promotes survival and growth of cells that have incorporated the targeting vector, either at the target site or at a random location within the genome. Inclusion of the "negative" selectable marker helps to eliminate most of the cells that have incorporated the targeting vector at a random location.

The positive marker, usually a *neomycin-resistance* (*neo*^{r}) gene, is positioned so that it will be flanked by DNA also present in the target gene. The negative marker, typically the *thymidine kinase* (*tk*) gene from a herpesvirus, is attached to one end of the targeting vector [*see illustration on pages 36 and 37*]. When homologous recombination occurs, the unchanged segments of the cloned gene, together with the *neo*^{r} gene sandwiched between them, replace the target sequence in the chromosome. But the *tk* gene, lying outside the zone of matching sequences, does not enter the chromosome and is degraded by the cell. In contrast, when cells randomly insert the targeting vector, they stitch the entire vector, complete with the *tk* gene, into the DNA. When no insertion occurs, the vector and both its markers are lost.

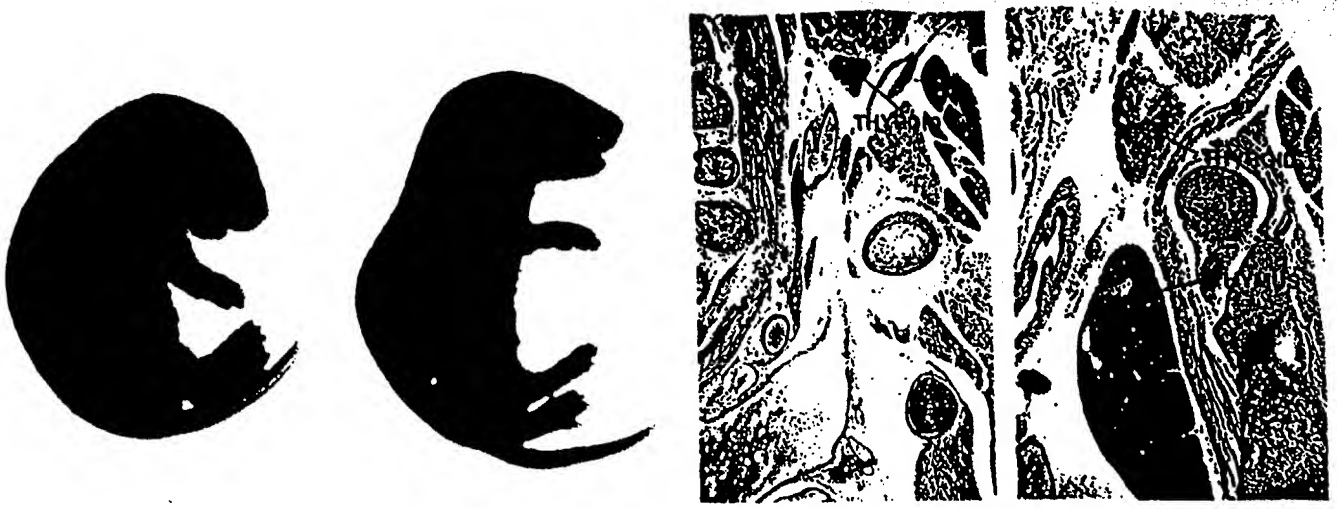
We do not have to examine the DNA directly to identify these different outcomes. Instead we grow the cells in a medium containing two drugs, an analogue of neomycin called G418 and the antiherpes drug ganciclovir. G418 kills cells that lack the protective *neo*^{r} gene in their chromosomes, namely, those that have failed to integrate vector DNA. But it allows cells that carry either random or targeted insertions to survive and grow. Concurrently the ganciclovir kills any cells that carry the herpes *tk* gene, namely, those that harbor a random insertion. In the end, virtually the only surviving cells are those bearing the targeted insertion (cells possessing the "positive selectable" *neo*^{r} gene and lacking the "negative selectable" *tk* gene).

By 1984 we had shown that it was possible to target specific genes in cultured mouse cells. We were then ready to extend the technology to alter the genome of living mice. To accomplish this aim, we used special cells developed in 1981 by Matthew H. Kaufman and Martin J. Evans of the University of Cambridge. These cells are embryo-derived stem (ES) cells. Such cells are obtained from an early mouse embryo. They can be cultured in petri dishes indefinitely, and they are pluripotent: capable of giving rise to all cell types.

In brief, by the procedure described earlier, we produce ES cells known to carry a targeted mutation in one copy of a chosen gene. Then we put the ES cells into early mouse embryos, which are allowed to develop to term. Some of the resulting mice, when mature, will produce sperm derived from the ES cells. By mating such mice to normal mice, we generate offspring that are heterozygous for the mutation—they carry the mutation in one of the two copies of the gene in every cell.

These heterozygotes will be healthy in most instances, because their second, undamaged copy of the gene will still be functioning properly. But mating of these heterozygotes to brothers or sisters bearing the same mutation yields homozygotes: animals carrying the targeted mutation in both copies of the gene. Such animals will display abnormalities that will reveal the normal functions of the target gene in all their tissues.

Of course, the procedure is more easily summarized than carried out. To actually do the work, we begin by injecting our modified ES cells into blastocyst-stage embryos, which have not yet become attached to the mother's uterus. Because we depend on coat color to indicate whether the procedure is going according to plan, we choose blas-



NEWBORN mouse (*above, left*) carries a targeted mutation in both copies of a gene called *HoxA-3*. Consequently, its body is more curved than that of a normal newborn (*second from left*). Tissue specimens from mutant (*center right*) and normal (*far right*) mice reveal that such mutants also lack a thymus and have an unusually small thyroid gland. These disorders and others indicate that the *HoxA-3* gene is needed for development of tissues and organs that originate in a narrow strip of cells (*colored band in drawing*) present in young embryos.

tocysts that would normally develop into pups bearing a different coat color than is found in pups produced by the mouse strain from which the ES cells are obtained.

The stem cells are isolated from a brown mouse carrying two copies of the *agouti* gene. This gene, even when present in a single copy, produces brown coloring by causing yellow pigment to be laid down next to black pigment in the hair shaft. (Production of the pigments themselves is under the control of other genes.) Hence, we typically select blastocysts that would normally develop into black mice. (Mice acquire black coats when the *agouti* gene inherited from both parents is defective.) Then we allow the embryo, containing the modified ES cells, to grow to term in a surrogate mother.

If all goes well, the altered ES cells reproduce repeatedly during this time, passing complete copies of all their genes to their daughter cells. These cells mix with those of the embryo and contribute to the formation of most mouse tissues. As a result, the newborns are chimeras: they are composed of cells derived both from the foreign ES cells and from the original embryo. We readily identify such chimeras by observing broad swatches of brown coloring in their otherwise black coats. If the animals bore no ES-derived cells, they would be completely black because of their lack of functional *agouti* genes.

By merely looking at the chimeras, though, we cannot determine whether the ES cells gave rise to germ cells, the vehicle through which the targeted mutation is passed to future generations. We find that out only when we move to the next stage: producing heterozygous mice harboring one copy of the mutation in all their cells. To generate such animals, we mate chimeric male mice to black female mice lacking the *agouti* gene. An offspring will be brown if the sperm that fertilized the egg was derived from ES cells (because all such sperm carry the *agouti* gene). An offspring will be black if the sperm derived from the original blastocyst cells (which lack functional *agouti* genes).

Consequently, when we see brown pups, we know that the genes carried by ES cells made their way to these offspring. We can then think about setting up matings between heterozygous siblings in order to produce mice with two defective copies of the target gene. First, though, we must discern which of the brown pups carry a copy of the mutated gene. This we do by examining their DNA directly for the targeted mutation. When matings are set up between heterozygous siblings, one in four of the offspring will have two defective copies of the gene. We pick out the homozygotes by again analyzing DNA directly, this time looking for the presence of two copies of the targeted mutation. These animals are then examined carefully for any anatomical, physiological or behavioral anomalies that can provide clues to the functions of the disrupted gene. The total procedure from cloning a gene to generating mice with a targeted mutation in that gene takes approximately one year.

Laboratories all around the world are now applying gene targeting in mice to study an array of biological problems. Since 1989, more than 250 strains carrying selected genetic defects have been produced. A few examples of the emerging findings should illustrate the kinds of insights these animals can provide.

In my own laboratory, we have been exploring the functions of homeotic, or *Hox*, genes. These genes serve as master switches ensuring that different parts of the body, such as the limbs, the organs, and parts of the head, form in the appropriate places and take on the correct shapes. Studies of homeotic genes in *Drosophila* have yielded important clues to their activities [see "The Molecular Architects of Body Design," by William McGinnis and Michael Kuziora; SCIENTIFIC AMERICAN, February]. Yet many questions remain. For instance, *D. melanogaster* has o eight *Hox* genes, whereas mice and mans each have 38. Presumably, pansion of the *Hox* family played a · ical part in the evolutionary prog sion from invertebrates to vertebr supplying extra machinery needec a more complex body. Precisely · do all 38 genes do?

Before gene targeting became able, there was no way to answer questions, because no one had mice or humans with mutations i of the 38 *Hox* genes. My colleagu I are now embarking on a syste

effort to establish the function of the individual *Hox* genes. Later we will attempt to identify how these genes form an interactive network to direct the formation of our bodies.

As part of this program, we have discovered that targeted disruption of the *HoxA-3* gene leads to multiple defects. Mice carrying two mutated copies of the gene die at birth from cardiovascular dysfunction brought on by incomplete development of the heart and the major blood vessels issuing from it. These mice are also born with aberrations in many other tissues, including the thymus and parathyroid (which are missing), the thyroid gland, the bone and cartilage of the lower head, and the connective tissue, muscle and cartilage of the throat.

These abnormalities are diverse but share one striking commonality: the affected tissues all descend from cells that were originally clustered in a narrow zone in the upper part of the developing embryo. The rudiments of the heart, for instance, are located in this region before the heart takes up its more posterior location in the chest. It seems, then, that the assignment of the *HoxA-3* gene is to oversee construction of many of the tissues and organs that originate in this narrow region.

Unexpectedly, the disorder produced by knocking out the mouse *HoxA-3* gene mimics that found in an inherited human disease known as Di George syndrome. Chromosomal analysis of patients shows that the human *HoxA-3* gene is not the culprit; victims display genetic damage on a chromosome distinct from that housing *HoxA-3*. We now know, however, that the gene responsible for the syndrome acts by interfering either with activation of the *HoxA-3* gene or with the events set in motion by the *HoxA-3* gene. Also, a mouse model for the disease is now available and may eventually provide clues to treatment. This unanticipated benefit underscores once again the value of basic research: findings born of curiosity often lead to highly practical applications.

Like developmental biologists, immunologists have also benefited from gene targeting. They are now applying this technology to decipher the individual responsibilities of well over 50 genes that influence the development and operation of the body's two foremost classes of defensive cells—*B* and *T* lymphocytes.

Cancer researchers are excited by the technique as well. Often investigators know that mutations in a particular gene are common in one or more tumor types, but they do not know the normal role of the gene. Discovery of that role using our knockout technology can help to reveal how the mutant form of the gene contributes to malignancy.

The *p53* tumor suppressor gene offers a case in point. Tumor suppressor genes are ones whose inactivation contributes to the development or progression of cancer. Mutations in the *p53* gene are found in perhaps 80 percent of all human cancers, but until recently the precise responsibilities of the normal gene were obscure. The analysis of mice homozygous for a targeted disruption of *p53* indicated that *p53* probably acts as a watchdog that blocks healthy cells from dividing until they have repaired any damaged DNA that is present in the cell. Such damage often occurs in cells as a consequence of the frequent environmental insults to which we are subjected. The loss of functional *p53* genes eliminates this safeguard, allowing damaged DNA to be passed to daughter cells, where it participates in formation of cancers.

Many other diseases will be amenable to study by gene targeting. More than 5,000 human disorders have been attributed to genetic defects. As the genes and mutations for the disorders are identified, workers can create precisely the same mutations in mice. The mouse models, in turn, should make it possible to trace in detail the events leading from the malfunctioning of a gene to the manifestation of disease. A deeper understanding of the molecular pathology of the disease should permit the development of more effective therapies. Among the models now being constructed are mice with different mutations in the cystic fibrosis gene.

The study of atherosclerosis, a leading cause of strokes and heart attacks, is also beginning to involve gene targeting. In contrast to cystic fibrosis, atherosclerosis is not caused by mutations in a single gene. Defects in a number of genes combine with environmental factors to promote the buildup of plaque in arteries. Nevertheless, promising mouse models have been made by alterating genes known to be involved in the processing of triglycerides and cholesterol. I also anticipate that mouse models for hypertension, another culprit in heart disease and stroke, will soon be developed, now that genes thought to participate in its development are being identified.

As understanding of the genetic contribution to disease increases, so will the desire to correct the defects by gene therapy. At the moment, the techniques used for gene therapy rely on random insertion of healthy genes into chromosomes, to compensate for the damaged version. But the inserted genes often do not function as effectively as they would if they occupied their assigned places on the chromosome. In principle, gene targeting can provide a solution to this problem. Yet, before it can be used to correct the defective gene in a patient's tissue, investigators may need to establish cultures of cells able to participate in formation of that tissue in adults. Such cells, which like the ES cells in our studies are termed stem cells, are known to be present in bone marrow, liver, lungs, skin, intestines and other tissues. But research into ways to isolate and culture these cells is still in its infancy.

Before the technical hurdles to broad application of our methods in gene therapy are surmounted, gene targeting will find common usage in the study of mammalian neurobiology. Already mice have been prepared with targeted mutations that alter their ability to learn. As increasing numbers of neural-specific genes are identified, the pace of this research will surely intensify.

We can anticipate continued improvements in gene-targeting technology, but it has already created opportunities to manipulate the mammalian genome in ways that were unimaginable even a few years ago. To significantly aid in deciphering the mechanisms underlying such complex processes as development or learning in mammals, researchers will have to call on every bit of their available ingenuity, carefully deciding which genes to alter and modifying those genes in ways that will bring forth informative answers. Gene targeting opens a broad range of possibilities for genetic manipulations, the limitations of which will be set only by the creative limits of our collective imagination.

FURTHER READING

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Use of Transgenic and Knockout Strategies in Mice

By Michael J. Ryan and Curt D. Sigmund

Over 2 decades ago mouse models were generated with an exogenous gene integrated into its genome to create the first transgenic mice. Since that time, new methods have been developed and old methods improved, allowing investigators more flexibility to answer important questions about physiology and gene function. Transgenic and knockout technology have been particularly useful in the kidney as various transgenic mouse models have been successfully generated to gain a better understanding of renal physiology at the gene level. Now with the sequencing of mammalian genomes at the forefront of science, the need for transgenic technology to understand gene function in the context of the whole animal will become increasingly more important. Therefore, this article focuses on some of the strategies that can be used when generating transgenic mouse models.

A GREAT DEAL of excitement was recently generated within the scientific community and general population when Celera Genomics and the Human Genome Project, directed by the National Institutes of Health, completed the draft sequence of the human genome.[1,2] This announcement sparked many to wonder about the potential impact this accomplishment could ultimately have on the diagnosis and treatment of disease as well as understanding basic functions of the human body. In addition to the human sequence, investigators have focused on mapping genomes of other species, such as mouse and drosophila, which have been useful for investigating many physiologically relevant questions. Unfortunately, merely having knowledge of the gene sequence does not automatically confer the knowledge to treat and cure disease. In fact, sequencing these genomes is just the initial step toward a better understanding of genes and their physiologic function. The next obvious step to fully use the data from these genome databases is to understand what protein is encoded by each of these sequences. Although, indeed, this is the focus of many investigators, the physiologic role of each gene and its protein will not be realized until they are examined in the context of the whole animal.

The idea of introducing a foreign gene into a mouse, or transgenics, was first proposed by Gordon and Ruddle,[3] and has proven to be an extremely useful technique to study the role of specific genes in animal models. Given the recent deciphering of the human and murine genetic codes, this technology should continue to prove beneficial to understanding gene function at a physiologic level for many years. The mouse is an ideal animal system for which to make transgenic models, given the relative ease for obtaining germline transmission of a transgene as well as their large litters and relatively short gestation time. This article outlines the general concepts of making transgenic and knockout mice and some of the various strategies that are currently being used to better regulate transgene expression in these mice. There are 2 major approaches to transgenic technology, additive transgenesis and gene targeting.

ADDITIVE TRANSGENESIS

A transgenic model is generated when a (trans) gene is incorporated into the mouse genome. In the case of additive transgenesis, the transgene is microinjected into the pronucleus of a fertilized mouse egg that is subsequently implanted into the oviduct of pseudopregnant female mice. The transgene typically forms concatamers that insert randomly into the genome.[4] Although it is now routine to fuse a complementary DNA (cDNA) to a cell-specific promoter, full-length genomic constructs containing the genes' endogenous promoter are also routinely used. For example, we previously generated models expressing the human renin and human angiotensinogen transgenes from genomic segments containing their endogenous promoter and all exons and introns.[5,6] Fusing a

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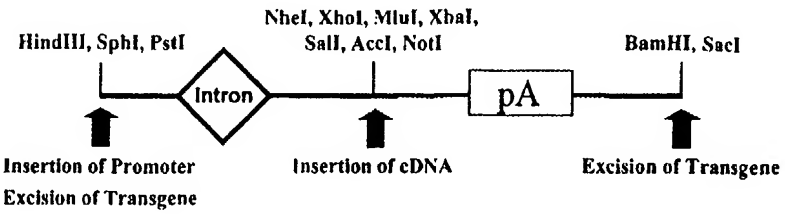

Fig 1. Schematic map of pSTEC-1 vector. The intron/SV40 poly A fragment was obtained from the plasmid pCI (Promega, Madison, WI). Only unique restriction enzyme sites are shown. For a more detailed map see Stec et al.[15]

cDNA to a highly restrictive cell-specific promoter provides a tool for the investigator to target expression of a transgene to a specific cell type. For example, we successfully used the kidney androgen-regulated promoter (KAP) to overexpress human angiotensinogen in renal proximal tubule cells, and the glial fibrillary acidic protein (GFAP) promoter to target the same gene to astrocytes in the central nervous system.[7,8] These models have been useful for investigating the role of tissue renin-angiotensin systems in blood pressure regulation.[8,9] In addition to the KAP promoter, there are other promoters that can be used to target unique renal cell types in transgenic mice including sodium hydrogen exchanger (proximal tubule), sodium potassium 2-chloride transporter (thick ascending loop of Henle), kidney-specific cadherin promoter (tubular epithelial cells), nephrin promoter (podocytes), and the aquaporin-2 promoter (principal cells of collecting duct).[10-14]

The promoter is only 1 of the components of a transgene. Typically the promoter is followed by an intronic sequence, a cDNA encoding the protein of interest, and a polyadenylation (poly A) sequence. To facilitate the generation of transgenes suitable for microinjection our laboratory designed a vector, pSTEC-1 (Fig 1), as a general backbone allowing the insertion of a cell-specific promoter and a cDNA.[15] The vector provides a chimeric intron and poly A site, which are very important because they encourage greater transgene transcription and messenger RNA (mRNA) processing.[16]

The generation of transgenic mice by pronuclear injection has yielded many important transgenic models. However, as with any model there are both advantages and potential limitations. The primary advantages to this technique are the speed at which a transgenic model can be generated and the high-level transgene expression that can be attained. Many universities now have transgenic core facilities that can make transgenic mice once provided with a transgene construct. Disadvantages include differences in transgene copy number and position of integration in the genome. Indeed, there is essentially no control over the number of transgenes that integrate into the genome. Although there is generally no direct correlation between transgene copy number and the level of expression, high-level supraphysiologic expression of transgene is possible, calling into question the physiologic relevance of such a model.

In addition to transgene copy number, the location of integration can have dramatic effects on the expression of a transgene. Called the *position effect*, transcriptional regulatory signals at or near the insertion site can strongly influence your transgene, even impart a new set of instructions. Such epigenetic influences can strongly modulate the transgene in a way distinctly different than normally encountered by a gene at its endogenous locus. In some cases the epigenetic influences on the transgene can be eliminated by dramatically increasing the size of the transgene. For example, though 2 previous transgenic mouse models expressing the human renin gene, 1 containing 900 bp of 5' flanking sequence and the other with 149 bp of 5' sequence, exhibited modest position effects,[6,17] a newer model containing a much larger 5' flanking sequence (75,000 bp) was immune to these effects and exhibited expression that was proportional to copy number.[18] In addition, the substantial size of the upstream sequence resulted in a much more restricted tissue distribution of expression and very tight regulation in response to physiologic cues.

Inducible Expression Systems

The choice of a cell-specific promoter in a transgenic experiment provides control over the spatial distribution of transgene expression, but unless the promoter has attractive temporal properties, there may be little control over when the gene is expressed. In the case of the KAP promoter, the

androgen dependency of the promoter provides a mechanism to turn the transgene on and off in response to exogenous androgen in females or androgen-receptor blockers in males.[19] Unfortunately, most promoters do not have similarly attractive temporal properties. Despite this, a number of ligand-based systems have been developed to provide an element of temporal control.

The tetracycline inducible system provides the investigator with control to turn the transgene on or off.[20] This method requires the generation of 2 separate transgenic models. The first model is engineered to express a tetracycline-controlled transactivator protein (tTA, tet-off; or rtTA, tet-on) from the desired cell-specific promoter (transactivator construct). The second transgenic mouse is designed with a transactivator-responsive promoter upstream of the cDNA of interest (trans-responder construct). The double transgenic mouse that results from the breeding of these 2 models will have both transgene constructs in all cells, but will only express the trans-responder construct in the correct cell types depending on the presence of absence of tetracycline or its derivative, doxycycline.

Other types of inducible systems for the design of transgenic models are built on the use of hormones such as mifepristone (RU486) or the Drosophila hormone, ecdysone, or its synthetic analog muristerone.[21,22] Like the tetracycline-inducible system, hormonal control of a transgene requires 2 transgenic models. In the case of the RU486-inducible system, GAL4 DNA binding sites and a minimal promoter are located upstream of the cDNA of interest. The second transgenic model expresses a truncated progesterone receptor under the control of a tissue-specific promoter. This progesterone receptor is not activated by progesterone, but by RU486, so that in its presence, the receptor is activated and binds to the GAL4 DNA binding site to activate transcription.

The ecdysone system adapted from Drosophila uses a trans-responder construct containing an ecdysone response element and minimal promoter upstream of the gene of interest. The trans-activator construct contains a modified ecdysone receptor (ER, that readily forms a heterodimer with the Retinoid X Receptor [RXR]), under the control of a cell-specific promoter. When muristerone is present, ER and RXR form a heterodimer, bind to the ecdysone response element upstream of the target gene, and initiate transcription.

GENE TARGETING

Gene targeting is a very powerful method for manipulating the mouse genome. This method uses mouse embryonic stem cells (ES) derived from the inner cell mass of a blastocyst-stage embryo (Fig 2). Several ES cell lines have been established that

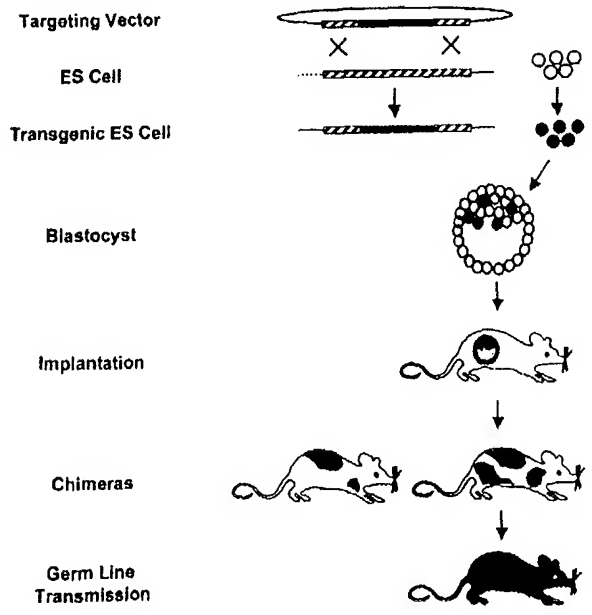

Fig 2. Schematic of gene targeting. Gene targeting requires the use of a targeting vector containing the modified gene (solid) flanked by regions of homology (hatched). After a successful recombination event, the transgenic ES cells are injected into a blastocyst and subsequently implanted into the mouse. The offspring are chimeric mice each with a different extent of chimerism. Chimeric mice are bred to establish stable transgenic lines. For more details see Capecchi[24] and Bronson and Smithies.[62]

retain their totipotency, that is, the ability to develop into all adult tissues.[23] ES cells can first be genetically modified via homologous recombination and then reinserted into a host blastocyst to transmit the modified genome to offspring.[24,25] The first offspring is a chimeric mouse that contains cells from the host blastocyst as well as the manipulated ES cells. Chimeric mice are bred to generate transgenic founders that carry the manipulated genome in all cells. The main strength of this approach lies in the flexibility of the manipulations that can be accomplished (see later), and the elimination of copy number and position effects. The primary limitation is that gene targeting is more time consuming and more technically challenging than microinjection. However, as stated earlier, the development of core facilities is eliminating the technical hurdles required to generate gene targeted mice.

Knockout Mice

To gain a better understanding of the function of a gene product, investigators have often turned to pharmacologic approaches to inhibit a gene or protein function. For example, pharmacologic inhibitors of various components of the renin-angiotensin system have been widely used to better understand this pathway. However, biologic systems are very complex and the inhibition of a desired target is only as good as the specificity of the pharmacologic antagonist, or our ability to target the agent to a specific tissue. Moreover, these agents often have biphasic effects depending on the dose of the drug or the model system to which it is being applied. Gene targeting technology may be used to circumvent such problems by providing a targeted specificity that may not be possible with some inhibitors. Nevertheless, the combination of gene targeting along with pharmacologic intervention can be a very powerful tool to dissect a biologic system. For example, we recently used direct administration of angiotensin-II and an AT1-receptor antagonist to the brain of AT1A and AT1B knockout mice to show that the pressor actions of central angiotensin-II are mediated by AT1A, whereas the dipsogenic actions of central angiotensin-II are mediated by AT1B.[26]

To make a knockout mouse, a targeting vector, which contains a homologous portion of the gene of interest, is engineered to contain a neomycin resistance (neo) cassette (or some other selectable marker) in place of a portion of the gene (ie, an important coding exon). After homologous recombination, the neo gene acts as the mutagen, thus, destroying the coding potential of the gene. On breeding of the altered genome to homozygosity, the function of the gene in question can be ascertained. Of course, though this strategy is extremely powerful, complete loss of gene function often results in a lethal phenotype. Loss of function of each gene in the renin-angiotensin system leads to mice that cannot survive past weaning.[27-32] Moreover, despite the power of the approach, it is critical to recognize that the resultant phenotype, death or other, may be the result of loss of gene function at the earliest stage of embryonic development. One mechanism to help elucidate the cause of death or dysfunction in these mice is through a combination of the use of transgenic-mediated rescue in knockout mice. We applied this approach to examine the cause of death in angiotensinogen knockout mice and discovered, to our surprise, that the death and renal abnormalities are not caused by the absence of angiotensinogen synthesis in the kidney.[33,34]

Cre-LoxP

As indicated earlier, the loss of gene function in all cells can result in a lethal phenotype. For genes that exhibit a complex pattern of tissue-specific expression, the lethality may be caused by the loss of expression in 1 critical tissue during development. Therefore, it would be very attractive experimentally to be able to generate tissue-specific deletions of a gene. By using such an approach, an investigator can target a gene deletion to individual tissues one at a time. The Cre-loxP method provides investigators with the ability to control the spatial distribution of a gene deletion (Fig 3). This technique is based on the bacteriophage Cre-recombinase and location of crossing over sites (loxP).[35] Cre-recombinase is a 38 kd enzyme that causes recombination by recognizing loxP sites in genomic DNA. LoxP sites consist of 2 inverted repeats of 13 bp, separated by an 8-bp central core sequence. When Cre-recombinase is expressed in the presence of DNA containing a sequence flanked (or floxed) by loxP sites, the sequence between the sites is deleted leaving only 1 intact loxP site. One can therefore engineer a targeting vector to contain loxP sites in intronic sequence surrounding a critical coding exon of the gene.

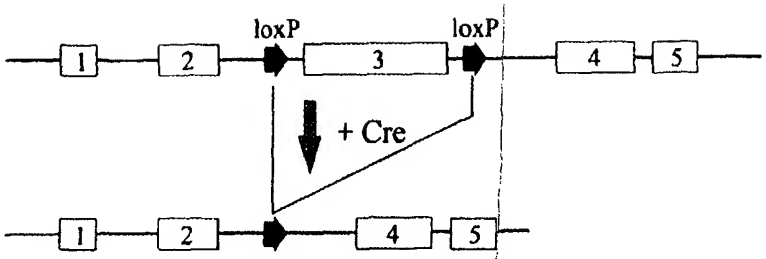

Fig 3. Schematic of Cre-loxP system. The targeted gene is designed with loxP sites flanking an important coding exon (open box). The addition of Cre-recombinase will result in the deletion of the flanked exon, disabling the gene. For more details see Stec and Sigmund.[53]

After homologous recombination, loxP sites will be transferred into the genome. Because introns are spliced out during transcriptional processing, the gene will function normally in the absence of Cre-recombinase. However, once Cre is introduced, the floxed-sequence will be permanently deleted rendering the gene inactive only in the tissues expressing Cre. Clearly, the specificity is in the delivery of Cre-recombinase to the proper cells and at the proper time.

Cre-recombinase can be delivered in a cell-specific manner by generating transgenic mice fusing a cell-specific promoter with Cre-recombinase.[36] In kidney, targeted expression of cre-recombinase to principal cells of the collecting duct under the control of the aquaporin-2 promoter has been reported.[12,37] Alternatively, as mentioned earlier, an element of both spatial and temporal control can be used with 1 of the ligand-based inducible expression systems.[38] Other new forms of Cre-recombinase activated only in the presence of certain ligands have also been developed.[39,40]

A second common way to introduce Cre-recombinase is through adenoviral delivery.[41] Although there is no real targeted specificity via this approach, it is worth noting that adenoviruses administered intravenously efficiently infect the liver, but only poorly infect other tissues. Indeed, our laboratory has shown the efficient liver-specific deletion of a floxed angiotensinogen transgene by delivery of an AdCre virus.[42]

Knock-In

The use of gene targeting does not end with the ability to knock out a gene. Homologous recombination in ES cells can also be used for the addition of exogenous DNA. In particular, this is most often used to insert the transgene at a specific locus within the genome, thus, controlling for both copy number and limiting positional effects of transgenesis. The overall methodology involved to generate a knock-in is very similar to that used to generate a knockout, with the main exception being the design of the targeting vector. In 1 use of the method, a target gene is inserted into the genome behind a cell-specific promoter. Chen et al[43] targeted Cre-recombinase into the myosin light chain 2v (MLC2v) genomic locus.[43] This locus was chosen because the promoter is expressed in the heart and in ventricular cardiomyocytes at the earliest stage of ventricular specification during development. Inserting Cre-recombinase into the MLC2v locus provided a means to obtain the same pattern of developmental- and cell-specific expression of Cre-recombinase without having any a priori knowledge as to how much of the MLC2v promoter would be required. Similarly, in the kidney, Pentz et al[44] used a strategy to insert green fluorescence protein (GFP) behind the Ren-1^d promoter to provide a sensitive marker of renin expression during development.[44] This provided a model similar to one developed by standard microinjection of a renin promoter–GFP fusion transgene.[45]

Our laboratory has recently used a modification of a method first described by Bronson et al[46] for inserting a gene in a single copy and at a defined locus in the genome.[46] We targeted the complete human angiotensinogen gene to the hypoxanthine phosphoribosyltransferase (HPRT) locus in the mouse genome by using direct selection in ES cells containing a partially deleted HPRT gene.[47] The targeting vector included the entire human angiotensinogen gene, sequences homologous upstream and downstream of the HPRT locus, and the missing portion of the HPRT gene. Successful recombination results in restoration of the HPRT gene (a selectable event) and inserts the human angiotensinogen gene upstream of HPRT. This highly efficient method of screening transgenic ES cell clones led to the production of 2 transgenic models that each have a single copy of the human angio-

tensinogen gene at a known locus in the genome, but which differ at positions in the gene that are thought to cause a differential predisposition to hypertension.[48] We are currently using this method to assess the physiologic significance of gene variation in this and several other genes associated with a genetic predisposition to hypertension. Other investigators have used the same approach to target the human haptoglobin gene to the apolipoprotein AI-CIII locus on chromosome 9, where they found a higher level of expression than at the HPRT locus.[49]

A CAUTIONARY NOTE

It is very important to recognize that each of the models described herein have some potential limitations and that no model will perfectly emulate a gene at its normal location in the genome. Moreover, in the end a phenotype observed in these experiments is not only the consequence of the manipulation made, but the genetic background of the animals being studied. Numerous differences in baseline phenotypes, such as blood pressure and tumor susceptibility, have been reported in different inbred strains of mice.[50] Although most ES cells used in gene targeting are derived from the 129 inbred mouse strain, they are reimplanted into C57BL/6 blastocysts and then bred with C57BL/6, thus, forming a mixed genetic background. Until 7 to 10 rounds (3–4 years) of successive backcross breeding is performed, it is crucial to use nontransgenic littermates as the control animals for any experiment to ensure that the experimental differences do not result from influences of genetic background. This is particularly important when one considers our recent data showing that the 129 strain has a genetic defect in relaxation of the aorta in response to an endothelial-dependent agonist.[51]

CONCLUSION

In this review, we have outlined several different strategies for designing transgenic mouse models. Since the advent of transgenic technology in the early 1980s, these methods have continually evolved to suit the needs of biomedical researchers. As a result of these powerful techniques we have been able to better understand the regulation of genes both at a physiologic and genetic level. Moreover, since the completion of the human genome sequence there will be a growing need to investigate the role of genes in animal systems so that the ultimate goal of treating disease can be met. Given the past and present successes of using transgenic and gene-targeting technology, it is clear that these methods will continue to allow investigators to learn about gene function in in vivo model systems.

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